Ultrasonic Methods for Analytical Determination of Pancreatic Enzyme Activities in Pharmaceutical Preparations

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“Die Neugier steht immer an erster Stelle
eines Problems, das gelöst werden will.”
(Galileo Galilei)

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LIST OF ABBREVIATIONS AND/OR TERMS

a	Specific concentration increment
API	Active Pharmaceutical Ingredient (Drug substance)
APM	Auxiliary processing module (Equipment of Microfluidizer)
ATR	Attenuated Total Reflectance
BAEE	N-Benzoyl-L-arginine ethyl ester hydrochloride
BB	Borate buffer
BSA	Bovine Serum Albumin
CDER	Center for Drug Evaluation and Research
CV	Coefficient of Variation
d	Length
ρ	Density
EDTA	Ethylenediamine-tetraacetic acid
E	Enzyme
EK	Enterokinase
EX-FTC	External Flow through cell
f	Frequency
FIA	Flow Injection Analysis
FIP	International Pharmaceutical Federation
FDA	Food Drug Administration
FTIR	Fourier transform infrared (Spectroscopy)
FTS	Flow Through System
HLB	Hydrophilic-lipophilic balance
HR-US	High-Resolution Ultrasonic Spectroscopy
IPC	In-process Control
k	Rate constant

κ	Adiabatic compressibility
IXC	Interaction chamber (Equipment of Microfluidizer)
LAM	Lipase Activating Mixture (Mixture from bile salts, Ph.Eur.)
LOV	Lab-on-valve
Mal	Maleic anhydride
MIR	Middle-infrared (Spectroscopy)
n	Integer number (e.g. of enzyme solutions)
NIR	Near-infrared (Spectroscopy)
p.a.	for analysis
PAT	Process Analytical Technology
PEI	Pancreatic Exocrine Insufficiency
Ph.Eur.	European Pharmacopoeia
P	Product
PP	Phosphate buffer
PPA	Porcine pancreatic amylase
rep	Replicate(s)
RM	Reference method
SIA	Sequential Injection Analysis
Stdev	Standard deviation
Std Work	Pancreas powder working standard (solution)
S	Substrate
t	Time
TCA	Trichloroacetic acid
TRIS	Tris(hydroxymethyl) aminomethane
Triton X 100	Polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether
US	Ultrasonic Spectroscopy
USP	United States Pharmacopoeia
UV/ Vis	Ultraviolet/ Visible (Spectroscopy)
v	Ultrasonic velocity
w	Concentration (mass fraction)
λ	Wavelength

1. INTRODUCTION

1.1 Use of Pancreas powder in medicine - history and presence

In the ancient world, the term *pán kréas* ("totally from meat") emerged. Although the position and the glandular constitution of the pancreas had been well known for a long time, the function was not really understood. Only a protective function of adjacent vessels was attributed to the pancreas by Aristoteles [384-322 B.C.] or a sustaining function by Galen [129-199 A.C.]. This undervaluation might be a reason for the lack of research in the following centuries (Kuhlmann, 1999). Opinions partially changed in the middle of the 17th century, when Moritz Hofmann and Johann Georg Wirsüng discovered the excretory duct without understanding its function. Few decades later, François de le Boë Sylvius argued that pancreas secretion intermingles with nutrition to serve the "*fermentatio*". Concurrently, the first pancreas secretion was obtained from living animals via a fistula (Kuhlmann, 1999). With the birth of endocrinology in the second half of the 18th century, *in-vitro* experiments with pancreas secretion were undertaken. Later, imitated digestive juices were prepared by Eberle (1834). However, it was not until the middle of the 19th century, when pancreas secretion was first described as the universal digestion juice. Claude Bernard considered the pancreas secretion important for the cleavage of saccharides, proteins as well as fats, in contrast to gastric juice or saliva. Furthermore, he showed that the active ingredient was precipitable with alcohol. The precipitate was named Pancreatin (Kuhlmann, 1999).

Fles (1864) successfully treated pancreas insufficiency in a patient by dispensing an extract of minced bovine pancreas to each meal. Langdon-Down achieved the same result with purchasable Pancreatin. However, criticism of purchasable Pancreatin was extensive. For example, Ewald (1880) regarded it as completely ineffective due to the loss of enzyme activity during stomach passage. At the beginning of the 20th century, the Rhenania AG (Aachen) launched a potent preparation containing Pancreatin partially associated with tannin, which was resistant to gastric juice *in vitro* (Pankreon®). Since the tannin was split off insufficiently due to the slightly alkaline pH in the duodenum, the high concentrations of active enzymes in Pankreon® instead of the association to tannin emerged as reason for the potency of the preparation *in vivo* (Kuhlmann, 1999). In order to ensure no release in the stomach (pH below 4) and an immediate

release of enzymes in the proximate duodenum (pH of 5.5 to 6), enteric-coated pancreatin preparations have been established soon after introduction of Pancreon® (Kuhlmann, 1999, Creon International Scientific Brochure, 2007).

Pancreatin is indicated in cases of Pancreatic Exocrine Insufficiency (PEI) as a consequence of chronic pancreatitis or pancreatectomy (e.g. due to alcohol abuse or cystic fibrosis), after gastric resection (e.g. due to gastric cancer) as well as after acute pancreatitis (Lankisch, 1999; Böhme *et al.*, 2006). Malfunction of pancreatic lipases is accompanied by steatorrhea owing to the diminished fat digestion (Ruyssen & Lauwers, 1978). Pancreatin eliminates the steatorrhea and its accessory symptoms, such as reduction of weight, meteorism and flatulence. If patients show persisting steatorrhea, a low duodenal pH may be the reason (Lankisch, 1999). Disturbances in protein digestion merely occur in severe cases of pancreatic insufficiency as pepsin ensures gastric proteolysis. Amylase defects are not considerable (Böhme *et al.*, 2006). Diabetis mellitus (Frier *et al.*, 1976; Semakula *et al.*, 1996) or Human Immunodeficiency Virsus Infection (Carroccio *et al.*, 2001) have also shown symptoms of PEI.

The dose of Pancreatin-containing preparations depends on the severity of PEI and the composition of the meal, especially the content of fat. In severe cases of steatorrhea, cystic fibrosis and after pancreas extirpation a dose of 0.5 up to 1 gram of Pancreatin per meal may be necessary (Ruyssen & Lauwers, 1978), a total loss of pancreatic function requires doses up to 2 grams of Pancreatin per meal (Böhme *et al.*, 2006).

1.2 Definition and properties

According to European Pharmacopoeia (Ph.Eur.), “Pancreas powder” is prepared from fresh or frozen mammalian pancreases and contains various enzymes having proteolytic, lipolytic and amylolytic activities. It is a slightly brown or cream colored, amorphous powder, which is incompletely soluble in water and practically insoluble in 96 % (v/v) ethanol (Ph. Eur.; Ryssen & Lauwers, 1978). The United States Pharmacopoeia (USP) differentiates two compounds in quality: “Pancreatin” and “Pancrelipase”. From here forth, the term “pancreas powder” will be used according to Ph.Eur. definition.

The dosage strength of a preparation containing pancreas powder is usually defined by its active lipase content as this is considered to be the most important and most sensitive digestive enzyme of the pancreas. In order to ensure product quality, the pharmacopoeias give analysis methods and specification limits for each of the three enzyme classes (proteases, lipases and amylases). Since enzyme mass is not related to drug effect, pancreatic enzyme activity with defined substrates is determined. Ph.Eur. methods are based on methods developed by the International Pharmaceutical Federation (FIP). Thus, Ph.Eur. and FIP use the same units. In spite of specifying the same substrates, USP methods vary for all three enzyme classes. The definition of one amylase or protease activity unit (USP-unit) is different whereas lipase activity according to USP is approx. equivalent to lipase activity given in Ph.Eur. units

(Böhme *et al.*, 2006; Creon International Scientific Brochure, 2007). Ph.Eur. and USP describe convention methods and thus a strict compliance with conditions described in the monographs is required to obtain comparable results. Relative enzyme activities are determined using a pancreas powder reference standard.

The minimum enzyme activity of the Active Pharmaceutical Ingredient (API) varies according to each Pharmacopoeia (Table 1).

Table 1. Enzyme activities in 1 mg of pancreas powder according to Ph.Eur. and USP. In order to compare the enzyme activities, USP-units were converted to Ph.Eur.-units based on conversion factors according to Martindale (1993). In the USP, free proteases are declared, while the Ph.Eur. refers to total proteases including free and zymogens of the proteases which are activated by enterokinase (n.l.t. = not less than, API = Active Pharmaceutical Ingredient).

	API	Lipase activity	Amylase activity	Protease activity (free)	Protease activity (total)
Ph.Eur. (Ph.Eur.-units/ mg)	Pancreas Powder	n.l.t. 15	n.l.t. 12	-	n.l.t. 1
USP (USP-units/ mg) [Ph.Eur.-units/ mg]	Pancreatin	n.l.t. 2 [2]	n.l.t. 25 [6]	n.l.t. 25 [0.4]	-
USP (USP-units/ mg) [Ph.Eur.-units/ mg]	Pancrelipase	n.l.t. 24 [24]	n.l.t. 100 [24]	n.l.t. 100 [1.6]	-

Lipolysis in the gastrointestinal tract is an interplay of different types of lipases, mainly gastric and pancreatic triacylglycerol lipases. In addition, lower proportions of pancreatic carboxylester lipase (equal to cholesterol esterase) and Phospholipase A_2 are present (Ruyssen & Lauwers, 1978). Except for carboxylester lipase, the other lipases have natural substrates which are water insoluble. Maximum activity is observed when the lipase is adsorbed to an oil/ water interface. Thus, it is the substrate amount available at the interface which represents the effective substrate concentration for the enzyme. Due to the heterogeneous reaction, digestion of lipids takes more time than the degradation of carbohydrates and proteins. The coprotein colipase optimizes pancreatic lipase activity by formation of a stable complex with triacylglycerol lipase enabling it to penetrate the interface (Creon International Scientific Brochure, 2007). Furthermore, the colipase neutralizes the inhibitory effect of high bile salt concentrations (Borgström & Erlanson, 1973). At optimum bile salt concentrations, they enlarge the water/ lipid interface and stabilize the substrate emulsion, leading to an increased velocity of the lipase reaction (Creon International Scientific Brochure, 2007).

Pancreatic lipases are described as *sn*-1,3-specific, which means that they hydrolyze triglycerides to 1,2-diglycerides and 2-monoglycerides (Ryssen & Lauwers, 1976). According to the pharmacopoeias, olive oil is often used as substrate to determine lipase activity in titration methods. Another substrate frequently used is triolein which leads to a tenfold higher activity than olive oil (Tietz *et al.*, 1989). However, the most commonly used substrate in lipase assays is still tributyrin (Beisson *et al.*, 2000), in spite of the negative strong odor of butyric acids.

According to Ph.Eur. and USP, lipase activity is determined as follows: The triglycerides of

olive oil, which is emulsified in a gum acacia and bile salts-containing solution, are cleaved to glycerol and free fatty acids. The fatty acids are titrated with sodium hydroxide solution at pH 9 (pH stat-method) and 37°C. The lipolytic activity of pancreas powder is determined by comparing the rate at which a suspension of pancreas powder hydrolyzes a substrate of olive oil emulsion with the rate at which a suspension of a pancreas powder reference standard hydrolyzes the same substrate under the same conditions (Ph.Eur.). One Ph.Eur.-unit and one USP-unit, respectively, of lipase activity is equivalent to the amount of pancreas powder that liberates 1.0 micro equivalent of acid per min at pH 9.0 and 37°C under the conditions of the assay for lipase activity described in Ph.Eur. and USP, respectively (Böhme *et al.*, 2006; USP).

α-Amylase, systematically named α-1,4-glucan-glucanohydrolase, is an endoamylase that hydrolyzes α-1,4-glucan links in polysaccharides which contain a minimum of three α-1,4-linked D-glucose units, e.g. starch (Ruyssen & Lauwers, 1976). Figure 1 shows the phases of the pharmacopoeia assays (Ph.Eur. and USP) to determine amylase activity in pancreas powder and pancreas powder-containing material. In addition to the titrations of samples and reference standards, blank values are required. The substrate starch and the enzymatic material are incubated for 10 min at 25°C. The enzyme reaction is stopped with hydrochloric acid. Upon addition of iodine and sodium hydroxide, sodium hypoiodite is formed (disproportionation of iodine) which oxidizes the released reducing end groups of the terminal glucose units. Additionally, iodine forms a blue/ violet clathrate with the non-cleaved starch. The excess iodine is then titrated with thiosulfate until colorless. The amylolytic activity of pancreas powder is determined by comparing the rate at which a suspension of pancreas powder hydrolyzes a substrate of starch solution with the rate at which a suspension of a pancreas powder reference standard hydrolyzes the same substrate under the same conditions (Ph.Eur.). One Ph.Eur.-unit and one USP-unit, respectively, of amylase activity is equivalent to the amount of pancreas

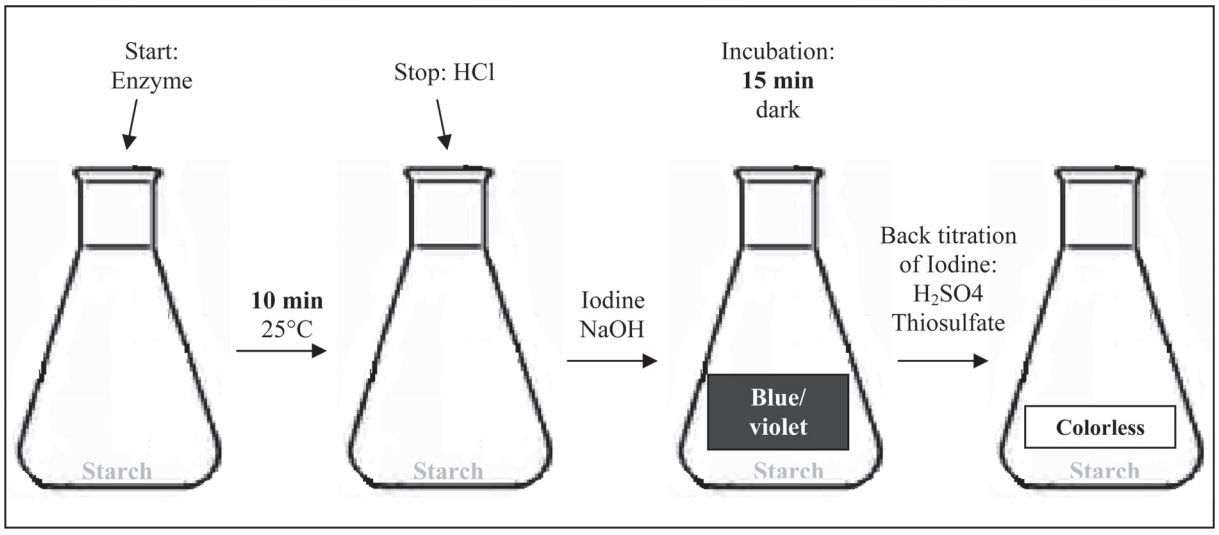

Figure 1. Phases of pharmacopoeia assays (Ph.Eur. and USP) to determine amylase activity in pancreas powder. The substrate starch and the enzyme are incubated for 10 min at 25°C. The enzyme reaction is stopped with hydrochloric acid. Upon addition of iodine and sodium hydroxide, sodium hypoiodite is formed (disproportionation of iodine) which oxidizes the released reducing end groups of the terminal glucose units. Additionally, iodine forms a blue/ violet clathrate with the non-cleaved starch. The excess iodine is titrated with thiosulfate until colorless.

powder that decomposes starch at an initial rate such that 1.0 micro equivalent (0.16 micro equivalent, respectively) of glycosidic linkage is hydrolyzed per minute under the assay conditions described in Ph.Eur. and USP, respectively (Böhme *et al.*, 2006; USP).

Pancreatic proteases are a mix of several endo- and exopeptidases, for example carboxypeptidase A and B, elastase, trypsin or chymotrypsin. In order to protect the pancreatic gland from self digestion, fresh pancreatic juice contains inactive pro-enzymes. Enzyme activation is started by enterokinase released from the intestinal mucosa, thrombin or trypsin itself. The zymogen trypsinogen is converted to trypsin, which in turn activates all other pro-enzymes (Ryssen & Lauwers, 1976; Creon International Scientific Brochure, 2007). In pancreas powder, the proteases are partially, but not completely activated. Thus, two kinds of proteolytic activities are distinguished:

- Free protease activity considers only the already activated proteases (USP)
- Total protease activity considers full activation after pre-incubation with enterokinase (Ph.Eur)

Casein (used in the pharmacopoeia assays) and hemoglobin are substrates commonly introduced in protease assays. Figure 2 shows the phases of the pharmacopoeia assays to determine free or total protease activity in pancreas powder. According to Ph.Eur., total protease activity is determined by initially activating the sample with enterokinase for 15 min at 35°C, whereas the sample is introduced without pre-incubation in the USP method to obtain free protease activity.

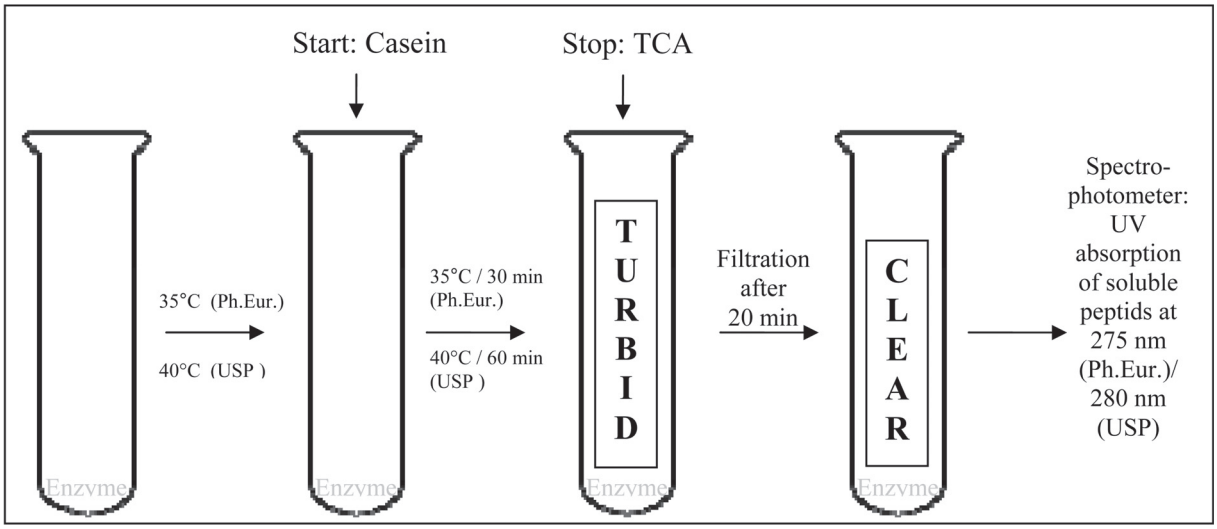

Figure 2. Phases of pharmacopoeia assays to determine total protease activity (Ph.Eur) or free protease activity (USP) in pancreas powder. Proteolysis is started by adding casein solution to the unactivated (USP) or activated (Ph.Eur.) enzyme. After incubation for 30 min (60 min USP) at 35°C (40°C USP), the reaction is stopped using trichloroacetic acid (TCA). Precipitated proteins are separated via filtration. Using a spectrophotometer, the UV absorption of soluble peptides remaining in the filtrate is measured at 275 nm (280 nm USP) (TCA = Trichloroacetic acid, Ph.Eur = European Pharmacopoeia, USP = United States Pharmacopoeia)

In addition to the determination of samples and reference standards, blank values are also required. All solutions are tempered. Proteolysis is started by adding casein solution. After incubation for 30 min (USP: 60 min) at 35°C (USP: 40°C) the reaction is stopped using trichloroacetic acid (TCA). Precipitated proteins are separated via filtration whereas soluble peptides remain in the filtrate. Using a spectrophotometer, the UV absorption in the filtrate is measured at 275 nm and 280 nm, respectively. The proteolytic activity of pancreas powder is determined by comparing the quantity of non-precipitable peptides released per minute from a substrate of casein solution with the quantity of such peptides released by a pancreas powder reference standard from the same substrate under the same conditions (Ph.Eur.). One unit of protease activity is equivalent to the amount of pancreas powder that hydrolyzes casein under the conditions of the assay for protease activity described in Ph.Eur. and USP, respectively, at an initial rate such that there is liberated per minute an amount of non-precipitable peptides that gives the same absorbance at 275 nm (280 nm USP) as 1 µmol and 15 nmol, respectively, of tyrosine (Böhme *et al.*, 2006; USP).

Ph.Eur. and USP describe different methods for determining trypsin activity. According to Ph.Eur., trypsin activity is determined by comparing the rate at which the trypsin-containing sample hydrolyzes N-benzoyl-L-arginine ethyl ester (BAEE) hydrochloride with the rate at which trypsin reference standard hydrolyzes the same substrate under the same conditions (Ph.Eur.). BAEE is cleaved to ethanol and benzoylarginine, which is titrated with sodium hydroxide solution at pH 8 (pH stat-method) and 25°C. In contrast, the USP method measures the hydrolysis of BAEE using a spectrophotometer. The hydrolysis of BAEE causes an increase in optical density at 253 nm (Sacks *et al.*, 1971).

Human pancreatic juice resembles porcine pancreatic secretion. Both pancreatic juices contain high portions of lipase and α-amylase. In contrast, more proteases are found in sheep and bovine pancreas (Peschke, 1991). Porcine pancreas accumulates as by-product in slaughterhouses in great quantities and thus it is conceivable that porcine pancreas is conveniently used for the manufacturing of pancreas powder. Except for muslime countries, bovine pancreas has become less important in the period of BSE and other infectious diseases (Böhme *et al.*, 2006). In order to obtain pancreas powder, raw material (fresh or frozen pancreas glands) is minced. Mechanical or proteolytic cell disruption then releases pancreatic enzymes into an aqueous, buffered medium. For example, hydrolysis is undertaken in an alkaline aqueous medium (GB 1328202, 1973) or in a neutral buffered medium (EP 0115023, 1984). After separation of solid components (cell debris, fibers), pancreatic enzymes are precipitated, for example by the addition of ethanol. The fat-containing supernatant is separated. The precipitate is washed and dried, yielding the API. Pancreas powder of a higher digestive power may be labeled as a whole-number multiple of the three minimum activities or diluted to the desired activity by mixing with lactose, sucrose or pancreas powder of lower digestive power (USP). Finally, the

enzyme mixture is formulated to either enteric-coated pellets or mini tablets, which are filled in gelatine capsules or sachets (drug product). Enzyme activity of the API depends on the origin of the glands (i.e. the strain and feed of the pigs) and thus there may be high variability between batches. Due to the sensitivity of pancreatic enzymes, the conditions of all manufacturing steps, such as composition of the extraction agent or temperature during drying, influence the final quality of the pancreas powder (Peschke, 1991; Böhme *et al.*, 2006).

1.3 Process Analytical Technology (PAT)

1.3.1 Definition and requirements

The quality of a pharmaceutical product (API and drug product) is influenced by the characteristics of the raw materials, as well as by the manufacturing process. In 2004, the Food and Drug Administration (FDA), specifically the FDA's Center for Drug Evaluation and Research (CDER) released guidelines for manufacturing companies to ensure quality of pharmaceutical products. In these guidelines, the term Process Analytical Technology (PAT) was defined as *"(...) a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e. during processing) of critical quality and performance attributes for raw and in-process materials and processes with the goal of ensuring final product quality. (...) The goal of PAT is to enhance understanding and control the manufacturing process, (...)"* (FDA, Guidance for Industrie: PAT – A Framework for Innovative Pharmaceutical Development, Manufacturing and Quality Assurance, 2004).

PAT leads to a better process understanding, which consequently results in the optimized use of resources. Furthermore, the continuous monitoring of the manufacturing process allows for early intervention upon failures. Thus, rejections of batches can be prevented. If a correlation between in-process and release testing can be established, release testing of the final product (API and drug product) may be minimized or eliminated.
The FDA identified the following tools as being qualified for implementation of PAT in the pharmaceutical industry:

- Process analyzers
- Process control tools
- Multivariate tools for design, data acquisition and analysis
- Continuous improvement and knowledge management tools

Non-destructiveness, low costs, short analysis times, real-time monitoring and the possibility for automation are general requirements of potential in-process analysis and control (IPC)

techniques. The method applied needs to be robust, discriminatory and able to identify failures. Furthermore, no or marginal sample preparation is favored.
Based on sampling methods and location of the analytical instrumentation, process analyzers are differentiated as follows (FDA, 2004; Kessler, 2006):

Off-line: After manual sampling, the sample is transported to a remote laboratory to be analyzed (discontinuous measurement).

At-line: After manual or (semi-)automated sampling, the sample is transported to an analyzer within the manufacturing area to be analyzed in close proximity to the process stream.

On-line: The sample is automatically diverted from the process and transferred to an automatic analyzer. Afterwards the sample may be returned to the process stream (by-pass).

In-line: The sample is not removed from the process stream. The probe head is directly located in the process stream (invasive) or only separated by a window (noninvasive) from the process stream.

In the following sections, process analysis, especially the determination of enzyme activities, is introduced in detail with respect to the manufacturing process of pancreas powder. As there is no need for sample taking, transport, preparation and disposal, the in-line measurement is generally the best form of process analysis. Simple in-line analytics of parameters, such as temperature, pH, weighing or flow, which represent process quality only indirectly can be easily adopted without disrupting the manufacturing process. In contrast, determination of enzyme activity (direct target) cannot be done by in-line analysis, as the addition of a substrate solution to the sample is required. Therefore, enzyme activities are mostly analyzed off-line, but at-line or on-line techniques (without by-pass) are also conceivable. A branch line from the product stream to an analyzer would be required, where the sample is merged with the substrate solution and an analyzer monitors the enzyme reaction, in real- time.

In view of PAT, enzyme assays should be adoptable for IPC samples (liquid or solid) and for final release of API and drug product. Even though all samples harbor several pancreatic enzyme types, one enzyme or enzyme group needs to be determined selectively. Liquid samples obtained from the beginning of the manufacturing process contain a lot of potentially disturbing substances in the sample matrix, such as fibers, ethanol, other proteins or fat. Consequently, those samples are assumed to be the worst case. For the purpose of developing a PAT method, the used technique should be applicable for the determination of pancreatic amylases, proteases and lipases. In contrast, the pharmacopoeias apply different methods to determine pancreatic

enzyme activities in pancreas powder: Amylase activity is determined in a redox titration, lipase activity in an acid base titration and protease activity by using UV absorption (1.2). Nevertheless, in order to maintain proximity to the pharmacopoeia methods, the same substrates (starch, casein, olive oil) should be used. They are complex substances of natural origin with varying specifications.

1.3.2 Analytical methods for determining pancreatic enzyme activities suitable for PAT approach

Pharmacopoeia methods are time-consuming due to multi-step-procedures and sample preparation (e.g. dilution steps). Methods for the determination of enzyme activity used as in-process control are rarely found in the literature. Kroner (1988) reviewed and screened analytical methods regarding their suitability for on-line analysis of enzymes. Considering samples of high turbidity, only the electrochemical indication methods (potentiometry, amperometry, conductometry) were deemed appropriate. These methods are universally applicable to indicate end points of titrations, but not to follow the whole course of an enzyme reaction. Many reports on PAT related to bioprocesses, particularly fermentation processes, are available (Arnold *et al.*, 2002; Lopes *et al.*, 2004; Känsäkoski *et al.*, 2006; Rathore *et al.*, 2010; Workman *et al.*, 2007). Such bioprocesses usually have higher demands on the used method compared to enzyme reactions considering e.g. temperature changes, matrix substances or agitation. In the following, several methods are illuminated with respect to their applicability to determine pancreatic enzyme activities on the one hand and their availability as in-process control on the other hand. Kasten (2008) reviewed generally used in-line process techniques, but not concerning enzymes. Relating to liquid and aqueous samples (substrate/ sample mixture), the following techniques are of special interest in the context of the digestive enzymes in pancreas powder:

- Near-infrared (NIR) transmission spectroscopy and Middle-infrared (MIR)/ Attenuated total reflectance (ATR) spectroscopy
- Ultraviolet- visible (UV/ Vis) spectroscopy
- Ultrasonic measurements

In reviews concerning PAT, applications of NIR and MIR spectroscopy are widely discussed. NIR spectroscopy uses the near-infrared region of the electromagnetic spectrum (0.8 to 2.5 µm) and is based on molecular overtone and combination vibrations of the basic vibration in the MIR region (2.5 to 20 µm) (Kasten, 2008). MIR absorption bands are typically ten to hundredfold stronger than the weak NIR absorption bands, due to the higher energy and lower coefficients of absorption by NIR. The weak absorption of NIR often allows direct analysis of strongly

absorbing and high light-scattering matrices, such as fermentation broths. Therefore, dilution steps, short optical path lengths or dispersion in non-absorbing matrices are not required in the majority of cases (Arnold *et al.*, 2002). However, NIR absorption bands are typically very broad (up to 100 nm band widths) and highly overlapped, leading to complex spectrums. In addition to improvements in instrumentation, multivariate data analysis (e.g. partial least squares (PLS) and principle components regression (PCR)) have been developed to filter the required information. Thus, correlations between changes in calibration spectrums and variations in the calibration samples (e.g. concentration of the components) are defined (Kasten, 2008). The most critical aspect is to ensure that calibration models are representative for future variations in the samples (Arnold *et al.*, 2002). Besides, the process calibration is very laborious. Due to the strong absorbance of water, particularly in the MIR region, the water content in fermentation samples may cause problems (Känsekoski *et al.*, 2006). Water influences can be avoided by using ATR (attenuated total reflectance) spectroscopy in the MIR region or Raman Spectroscopy (Känsäkoski *et al.*, 2006, Kasten, 2008).

Determination of enzyme activity using NIR or MIR can be figured as follows: The sample is first added to the substrate solution before the mixture is transferred to a spectrometer or testing probe. Spectrums are generated in defined time intervals. Chemometric analysis using reference calibration data would yield the change of substrate and product concentration.

The use of IR spectroscopy to determine enzyme activity is frequently described in the literature, but not in context of in-process control and PAT (Barth, 2007). Enzyme assays are carried out either in an organic environment (Thoenges & Barth, 2002) or in aqueous media using heavy water D_2O (Wright & Vanderkooi, 1997; Pacheco *et al.*, 2003; Karmali *et al.*, 2004). Walde & Luisi (1989) developed an assay for determining lipolysis using Fourier transform infrared spectroscopy (FTIR). The authors prepared Aerosol OT (Dioctyl sodium sulfosuccinate)-reversed micelles with triglyceride (C6-C10) and vegetable oil (e.g. olive oil) in isooctane. Lipases were of mammalian and fungal origin (e.g. porcine pancreatic lipase). FTIR spectra of the entire reaction mixture were recorded and followed over time. For quantitative analysis, the authors chose isosbestic points in the spectrum: Peak of the formed fatty acids (comparatively large changes during reaction) and of the fatty acid esters. They showed that hydrolysis of triglycerides can be followed continuously with high reproducibility (1-2 %). O'Connor & Cleverly (1994) investigated human milk lipase using similar reversed micelles. The substrate was triolein, and bile salts were added to the mixture. A strong dependence of the reaction rate on water content in the reversed micelles was observed. However, use of an organic environment is not completely comparable with the pharmacopoeia methods. Using ATR-FTIR, Snabe & Peterson (2001) investigated a method for monitoring enzymatic activity on surface-attached substrate films in an aqueous environment. They monitored hydrolysis of triolein by lipase (native *Fusarium solani pisi* cutinase) and degradation of starch by *Bacillus amyloliquefaciens* α-amylase over time. The method consists of three steps: a) substrate film application, b) hydration of the film and c) addition of the enzyme. The long time required for

film hydration (up to 40 min) was only necessary to monitor enzymatic activity on surface-attached substrate films. Thus, considarable shorter analysis times are assumed using ATR-FTIR for determination of pancreatic enzyme activities.
FTIR for determination of α-amylase activity in aqueous solutions of starch (2-6 % (w/v)) proposed by Krieg *et al.* (1995) appears to be the most interesting method. Hydrolysis was carried out at 37 °C taking samples at defined time intervals to record FTIR spectra. Difference spectra were determined by subtracting the spectrum recorded immediately after adding the enzyme to the starch solution from the ones recorded subsequently 5 to 120 min later. That way, unspecific absorption of the matrix can be eliminated, and thus the determination of enzyme activity in a complex matrix (human sera) was possible. The authors observed a linear correlation between amylase activity and spectra intensities evaluating the sum of the differences in absorption at two pairs of wave numbers (1072 - 1157 cm^{-1} and 1115 - 1018 cm^{-1}) after 20 min incubation. Such long incubation times are not sufficient for PAT purpose. Provided that shorter analysis times (e.g. 5 min) also led to significant changes in absorption, this method would imply a high potential for PAT. In principle, application of IR is conceivable based on Snabe & Peterson (2001) and Krieg *et al.* (1995).

In the UV/ Vis region of the electromagnetic spectrum, molecules undergo electronic transitions. Organic compounds with a high degree of conjugation (aromatics or unsaturated hydrocarbons) absorb in the UV/ Vis region. In order to determine pancreatic enzyme activity by UV/ Vis spectroscopy, either UV/ Vis-active substrates/ products (Pencreac'h *et al.*, 2002) or coupling with a second reaction that leads to a conjugated system (Rejeb *et al.*, 2004) is required. Mostly high dilutions and filtration steps are also necessary.
Turbidity measurements are another application of UV/ Vis spectroscopy. Turbidity is measured by determining the attenuation of light caused by scattering or absorption. Turbidimetric and nephelometric determinations of starch and amylopectin degradation have been described in the literature (Peralta & Reinhold, 1955; Shipe & Savory, 1972; Malkus *et al.*, 1977). However, these methods have not gained great importance (Lorentz, 1979). Lipase-catalyzed hydrolysis diminishes turbidity in olive oil or triolein emulsion. The turbidimetric or nephelometric determination of lipase activity, especially in serum, has been widely discussed in the literature and steadily improved (Rick & Hockeborn, 1982; Kannisto *et al.*, 1983; Tietz *et al.*, 1987). In the meantime, turbidimetric determination of lipase in clinical applications has been well established.
However, clinical samples often do not exhibit a linear increase or even lead to an increase of turbidity caused by matrix effects (Rick & Hockeborn, 1982; Kannisto *et al.*, 1983). Although the methods are simple, poor substrate stability and the necessary calibration curve due to the lack of absolute lipase activity values hampered a wide diffusion of turbidimetric methods (Brobst, 1997; Beisson *et al.*, 2000). Concerning lipase, the production of an emulsion with reproducable droplet size is expected to be difficult (Schelong *et al.*, 2001).

Kawai *et al.* (1999) developed a turbidimetric assay to determine protease activity in the presence of 2 % (w/v) casein. After 2 h incubation, the turbidities were measured at a wavelength of 595 nm. The authors evaluated their method as fast, easy and economical. However, coagulating proteases were not measurable due to turbidity changes. Calcium in the samples changed the turbidity and pH variation led to unreliable results.
Thus, turbidimetry has two important demands which may limit the use as PAT method: First, a very stable substrate preparation is required, which does not coagulate or precipitate during measurement. Secondly, side reactions such as precipitation of calcium soaps or a simultaneous enzyme reaction may not occur as they can adulterate the results.

In addition to electromagnetic waves, acoustic waves can be applied as an analytic tool. Acoustic signals in the audible frequency region of 16 Hz to 20 kHz are named audible sound, above 20 kHz they are called ultrasound and are inaudible for humans (DEGA, 2006). However, many animals utilize ultrasound for navigation, as well as to find and characterize their food. According to Povey & Mason (1998), main ultrasound applications can be divided into two categories, depending on power (intensity) and frequency of the adopted ultrasound:

- At low power and high frequencies (> 1 MHz) as analytical tool
- At high power and low frequencies (20 to 100 kHz) to assist processing

High-intensity ultrasound using extremely high power levels (up to 1,000 $W \cdot cm^{-2}$) causes physical disruptions in the sample or promote chemical reactions (McClements, 1995). High-intensity ultrasound has been established as a means of generating emulsions or enhancing cleaning and dissolving processes. More recently, the use of ultrasound for the inactivation or activation of enzymes has been described (Shaw & Gupta, 2008; Mawson *et al.*, 2011). Using a probe system with a diameter of 6 mm, Liu *et al.* (2011) observed that ultrasonic pre-treatment (40 - 140 W, 6 min) altered the conformation of porcine pancreatic lipase and enhanced the specific activity. Tian *et al.* (2004) found a decrease of trypsin activity with increasing power (20 kHz, 85 - 425 W/ cm^2). Using ultrasound at appropriate frequencies and intensity levels often leads to an enhancement of enzyme activity due to e.g. optimized mass-transfer (Mawson *et al.*, 2011).
Low-intensity ultrasound typically uses very small power levels (below 1 $W \cdot cm^{-2}$). It is non-destructive because no physical or chemical changes of the sample occur while the waves pass through. Due to destructiveness and the possible inhibiting or activating effect on enzymes at high powers, only ultrasound with low power is adaptable for determining enzyme activity. The feasibility to determine enzyme activity based on changes of ultrasonic velocity or attenuation caused by changes in the sample (e.g. substrate degradation) has often been reported in the literature (Povey & Rosenthal, 1984; Kudryashov *et al.*, 2003; Born *et al.*, 2009).

Several characteristics of low-intensity ultrasound make this technique a high-potential tool for the determination of different enzyme activities in API, IPC samples or drug product. It is fast, automatable and does not need any sample preparation as it can be applied to concentrated and turbid samples. Due to the real-time monitoring of the enzyme reaction, the enzyme activity is determined quickly. According to pharmacopoeia methods, additional reagents are needed e.g. to stop the enzyme reaction (protease: TCA) or to determine non-cleaved substrate or the reaction product (amylase: iodine, thiosulfate). For ultrasonic measurements such reagents are not required. In contrast to UV/ Vis spectroscopy, conjugated systems in the substrates are not required. Therefore, complex mixtures of substrates as those used for pharmacopoeia methods and turbid preparations can be applied. The use of complex substrates with high molecular weight can even be advantageous for the ultrasonic measurement due to multiple degradation and structural changes (Born *et al.*, 2009).

1.4 Low-intensity ultrasonic measurements

1.4.1 Basics

Ultrasonic Spectroscopy uses high-frequency acoustic waves propagating through the sample. In liquid media, ultrasound propagates as longitudinal sound. The direction of the pulse is parallel to the oscillation direction of the molecules and their arrangements resulting in a local compression and decompression of the media.

1.4.2 Ultrasonic parameters

The most important parameters measured in ultrasonic experiments are ultrasonic velocity and attenuation. Together with reflectance, they form the primary acoustic response.

The density and the compressibility of the medium determine ultrasonic velocity. Compressibility, which is the reciprocal bulk modulus, describes the reduction of volume by pressure. The Wood equation, also named Laplace equation (Nomura *et al.*, 1987), describes the relationship between the ultrasonic velocity and the material constants as follows:

$$v = \sqrt{\frac{1}{\kappa \cdot \rho}}$$

v Ultrasonic velocity (m/s)

κ Adiabatic compressibility (Pa^{-1})

ρ Density (kg/m^3)

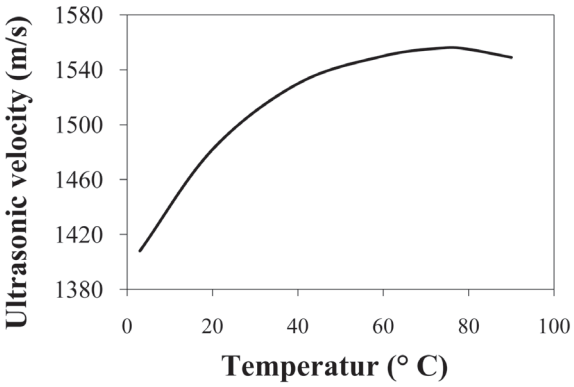

Figure 3. Temperature dependency of ultrasonic velocity in pure water. Maximum ultrasonic velocity is found at 74 °C (From: Povey, 1997).

This relationship is only effective for low-attenuation fluids (Coupland, 2004). The equation was complemented for dispersions of solid particles considering the volume fraction of the dispersed phase (Povey, 1997). Given its relation to density, the ultrasonic velocity in liquids depends on temperature. Unlike most fluids, the ultrasonic velocity in pure water and aqueous solutions increases with ascending temperature to a maximum at 74°C (Figure 3). Since the velocity in water varies by approx. 3 $m \cdot s^{-1} \cdot °C^{-1}$ at 20 °C an error in temperature of 0.1°C will produce an error in velocity of 0.3 $m \cdot s^{-1}$ (Povey, 1997). Ultrasonic velocity is only marginally affected by the frequency (Dukhin *et al.*, 2005). The ultrasonic velocity can be determined in two ways: Either the wavelength is determined at known frequency or the time needed for a wave to travel a known distance. (McClements, 1995).

Ultrasonic attenuation means the loss of energy and the decrease of amplitude of the ultrasonic wave as it passes through the sample. It is highly frequency-dependent and nearly unaffected by temperature changes (Dukhin *et al.*, 2005). The major causes of amplitude and phase alteration are absorption and scattering (McClements, 1995). Absorption implies the conversion of sound energy into heat, due to viscosity, thermal conductivity or molecular relaxation. Scattering occurs in heterogeneous materials. A discontinuity, such as a droplet in an emulsion or a particle in a suspension, causes scattering of the ultrasonic wave into directions which are different from that of the incident wave. In contrast to light detectors, ultrasound transducers cannot detect a signal in which the phases between different parts of the signal have been mixed up due to the phase sensitivity (Povey, 1997). This can lead to an extensive loss in the detected signal. Thus, measurements of attenuation are often less reliable than ultrasonic velocity measurements.

Dukhin *et al.* (2005) investigated the sensitivity of ultrasonic velocity and attenuation to chemical composition. Using the transmission pulse-technique, both parameters were measured in water with various concentrations of different chemicals. While ultrasonic velocity was shown to be very sensitive to addition of chemicals (approx. 2 % from 1500 $m \cdot s^{-1}$ to 1530 $m \cdot s^{-1}$ at a concentration of 0.3 $mol \cdot l^{-1}$ calcium chloride), the attenuation was less sensitive. It only changed significantly for highly charged ions and with concentrations varying by more than 0.1 $mol \cdot l^{-1}$. However, the authors observed a dramatic change in attenuation with composition of the dispersed phase during analysis of various dairy products. They concluded that sound velocity is useful for characterizing chemical compositions or reactions that occur on the molecular level, whereas attenuation is more suitable for characterizing effects related to heterogeneity and

phase composition. Other authors came to the same conclusions (McClements, 1995; Povey, 1997; Capote & Castro, 2007).

1.4.3 Experimental techniques and instrumentation

Ultrasonic instruments use either pulsed or continuous-wave ultrasound based on the form in which the ultrasound is applied to the sample (McClements, 1995; McClements, 1996; Kaatze *et al.*, 2008). McClements (1996) grouped the commonly used techniques into three categories (Figure 4). A fourth category can be defined by taking devices into account that are currently on the market (Table 2).

Table 2. Classification of ultrasonic techniques according to type of applied ultrasound (Letters in parentheses refer to Figure 4).

Pulsed ultrasound	Continuous-wave ultrasound
• Through Transmission Technique (A) • Pulse Echo Technique (B)	• Interferometric Technique (C) • Resonance Technique

A typical experimental configuration is composed of the measurement cell, which contains the sample, a pulse generator, an ultrasonic transducer and an oscilloscope or personal computer. A signal generator produces an electrical signal which is applied to the transducer converting the signal into ultrasound. The sound propagates through the sample in the measurement cell. Afterwards, the ultrasound is converted back into an electrical signal by the transducer acting as receiver. After digitalization by an analog-to-digital converter, the signal is displayed on the screen of an oscilloscope or personal computer (McClements, 1996). Transducers are generally composed of piezoelectric materials (e.g. lithium niobate or quartz crystals), which convert mechanical stress into an electrical voltage. Conversely, a mechanical deformation is produced when an electrical voltage is applied.

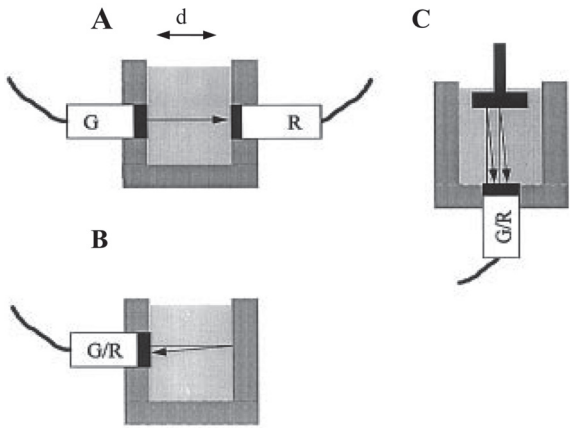

Figure 4. Principle setup of three of the most common measurement cells to measure ultrasonic properties. A shows the Through Transmission Technique, B shows the Pulse Echo Technique and C shows an Interferometer (G = Generator transducer, R = Receiver transducer, d = length of sample cell), for explanation see text (From: McClements, 1996)

For Through Transmission Technique (Figure 4, A), the measurement cell is located between two ultrasonic transducers. One transducer acts as generator, the second as a receiver. The ultrasonic velocity (v) is equal to the cell length (d) divided by time needed for travelling the distance (t) (Clements, 1996):

$$v = \frac{d}{t}$$

v	Ultrasonic velocity (m· s^{-1})
d	Length of sample cell (m)
t	Time (s)

Pulse Echo Technique (Figure 4, B) contains a single transducer that is used for generating and receiving the ultrasonic pulses. The ultrasound propagates through the sample and is reflected back to the transducer on the opposite wall. This technique is convenient for the determination of ultrasound in small volumes (Voleišienė & Voleišis, 2008). The time interval between the echo is the time required for the pulse to travel twice the cell length (Clements, 1995):

$$v = \frac{2\,d}{t}$$

v	Ultrasonic velocity (m· s^{-1})
d	Length of sample cell (m)
t	Time (s)

Interferometers (Figure 4, C) are also equipped with one single transducer, but together with a movable reflector plate. The generated ultrasonic wave is reflected back und forth between the generator and transducer inducing a standing wave in the sample. When the reflector plate is moved vertically through the sample, destructive and constructive interference occur leading to amplitude minima and maxima of the signals received by the transducer. This interferometric technique is suitable for precise absolute measurements (Voleišienė & Voleišis, 2008). However, it is more time consuming than pulsed techniques since measurements must be carried out separately for each frequency (McClements, 1996).

Resonances can also be generated in reverse. Instead of changing the path length while holding a constant frequency, the frequency is changed while a constant path length is ensured. Most modern resonance-based ultrasonic devices use this principle (Coupland, 2004). Here, ultrasound passes through the sample and generates a standing wave by being reflected on the other side (Figure 5). Resonance occurs when the length (distance between generator and analyzer) is an integer number of the half wave length:

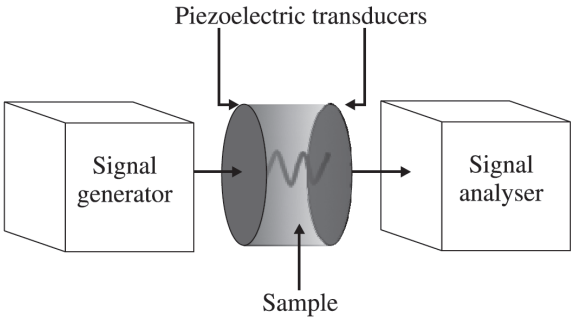

Figure 5. Principle setup of resonance-based ultrasonic devices. For explanation see text (From: Buckin *et al.*, 2003)

$$d = n \cdot \frac{\lambda}{2}$$

d Distance (cell length) [m]

λ Wavelength [m]

n Integer number

High amplitudes are caused by the interference phenomenon. By measuring resonance at high amplitudes, a higher precision is observed compared to pulsed techniques.
At least one resonance peak is defined prior to the measurement. The ultrasonic velocity is directly calculated from the frequency:

$$f_n = \frac{v}{\lambda}$$

f_n Resonance frequency [s^{-1}]

v Ultrasonic velocity [$m \cdot s^{-1}$]

λ Wavelength [m]

Thus each resonance peak is defined by the peak order number:

$$n = \frac{2\, d\, f_n}{v}$$

n Integer number (Peak order number)

d Distance (cell length) (m)

f_n Resonance frequency (s^{-1})

v Ultrasonic velocity ($m \cdot s^{-1}$)

Attenuation causes a broadening of the resonance peaks and can be calculated from the half-height width of the resonance peak (Coupland, 2004). In ultrasonic devices, the bandwith (3 dB) is generally used to calculate attenuation, in other words, the width at 70.71 % height of amplitude or the width at a point equal to the maximum amplitude divided by the square root of two.

Pulsed methods are the most widely used techniques due to their operating simplicity and rapidness of measurements (McClements, 1995). In addition, they have lower equipment expenditure and are often more robust compared to resonance methods (Coupland, 2004). However, as the path length of interactions of the ultrasonic field with the sample is virtually

increased by multiple reflections, continuous-wave techniques have a higher sensitivity. This allows high resolution measurements on small liquid volumes to become possible, at least in a limited frequency range (Kaatze *et al.*, 2008). Resonance cells can easily be constructed to measure volumes less than 1 ml while long path lengths (large samples) are required to measure changes in pulsed measurements. A pulsed method achieves maximum reproducibility within 0.1 $m \cdot s^{-1}$, whereas resonators can reach up to five orders of magnitude higher reproducibility. The precision of attenuation measurements is typically somewhat poorer for both techniques as compared to ultrasonic velocity measurements (Coupland, 2004).

Commercial instruments for high-resolution ultrasonic measurements are offered by TF Instruments GmbH (Ultrasonic Resonator Technology, URT) and Sonas Technologies Ltd. (High-Resolution Ultrasonic Spectroscopy, HR-US). Although the basic principle is identical, the instruments differ in cell volume, thermostat system and frequency range. Table 3 shows a comparison of both devices.

Table 3. Differences between commercially available high-resolution ultrasound-based instruments (* differential regime)

Technique	**URT**	**HR-US**
Device	**ResoScan Research System (Tf Instruments GmbH)**	**HR-US Spectrometer 102 P (Sonas Technologies Ltd.)**
Frequency range (MHz)	7 - 9	2 - 18
Cell volume (ml)	0.2	1.5
Control of temperature	Peltier element	External water bath
Range of temperature (°C)	5 - 85	-20 - 120
Resonance peak selection	automatic	manual
Accuracy for ultrasonic velocity ($m \cdot s^{-1}$)	0.001	0.0002*
Accuracy for ultrasonic attenuation (%)	1 - 3	0.2

The HR-US spectrometer provides a broader frequency range as compared to the ResoScan Research System. The ResoScan Research System facilitates the selection of the best resonance peak by evaluating the quality of the peaks concerning symmetry and amplitude during "initialization". In contrast, the operator has to ascertain voltage input (signal amplitude) and resonance peak manually using the HR-US spectrometer. The temperature control of the ResoScan Research System is carried out by a peltier element. The heating elements heat upon any temperature decrease, e.g. when the cell is opened. Therefore, the system quickly reaches the constant set temperature, as needed for reliable ultrasonic spectroscopic measurements. The cells in the HR-US102 P spectrometer are surrounded by flowing tempered water coming from a connected external water bath. The system cannot react on temperature changes in the cell block, leading to longer equilibration times. Due to heat loss during the circulation process, the real temperature in the cell block is not precisely known. Both devices are equipped with two

measurement cells. Therefore, reference measurements can be carried out. When performing enzyme kinetic measurements, both cells are filled with substrate solution. To one of the cells the enzyme solution is added. As only the change of velocity difference between both cells is considered, changes in temperature during measurement do not significantly affect the results, assuming same temperature variabilities in both cells. In the present study, measurements of ultrasonic velocity were possible with both devices although the instruments were run in non-conditioned rooms.

1.4.4 Determination of enzyme activity based on ultrasound

Enzymes are characterized by a high substrate and reaction specificity. They accelerate biochemical reactions by reducing the activation energy of the reaction. The enzyme binds the substrate in the active centre according to the "lock and key" model forming a stable enzyme-substrate complex. After chemical transformation the enzyme is regenerated and the products are released. A change of compressibility in the sample is assumed to lead to a change of ultrasonic velocity during enzyme reaction. According to Nölting & Sligar (1993), the adiabatic compressibility of e.g. proteins in aqueous solution is inter alia due to

- the intrinsic compressibility of the molecule
- the difference in compressibility in the hydration shell relative to bulk water.

Intrinsic compressibility results from an imperfect packing of the molecule. Relaxation leads to an increase of adiabatic compressibility, which is only significant if the relaxation is not much slower than the period of sound (Sarvazyan & Kharakoz, 1979). Hydration water shows less adiabatic compressibility as compared to bulk water leading to an increase of ultrasonic velocity. While in earlier papers (Gekko & Noguchi, 1979; Eden *et al.*, 1982) the prevailing view was that the compressibility of hydration water is close to the compressibility of ice, Kharakoz & Sarvazyan (1993) assessed that the compressibility of water in hydration shells is only 20 % lower than that of bulk water.

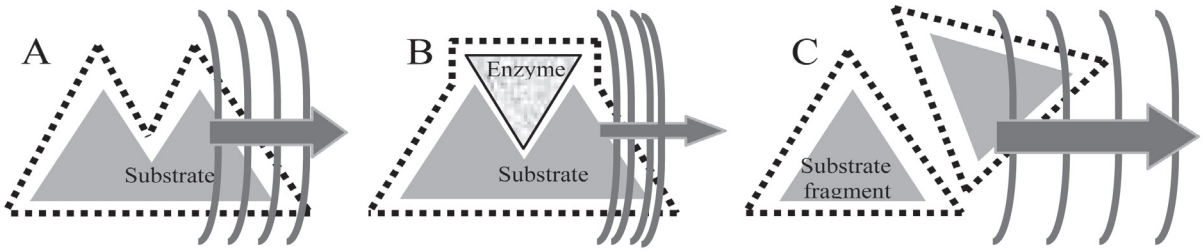

Figure 6. The change of ultrasonic velocity during enzyme reaction (schematically). Enzyme binding and substrate degradation lead to a change in extension of the surrounding hydration shells, which results in a change of ultrasonic velocity: **A** Properties of substrate solution determine the measured ultrasonic velocity. **B** After adding enzyme solution, the enzyme binds to the substrate leading to a release of hydration water and consequently to a reduction of ultrasonic velocity. **C** The substrate is cleaved to its single components leading to an increase of hydration water. Effects B and C interfere with each other. During hydrolysis reactions, a total increase of ultrasonic velocity is mostly observed (modified from TF-instruments).

Table 4. Ultrasonic determination of amylase, lipase and protease activity described in the literature.

Reference	Enzyme	Technique and Conditions	Conclusion
Povey & Rosenthal (1984)	Amylase	Pulse-echo technique: Determination of α-amylase activity in starch (5 %), 25 °C, measurement of ultrasonic velocity and attenuation (real-time)	Linear dependency of ultrasonic attenuation on amylase activity, ultrasonic velocity was unaffected
Kudryashov *et al.* (2003)	Amylase	HR-US: Determination of α-amylase activity in maltodextrin (1 %), 25 °C, measurement of ultrasonic velocity and attenuation (real-time)	Increase of ultrasonic velocity, decrease of ultrasonic attenuation during enzyme reaction; Recalculation of ultrasonic velocity curve into time dependency of converted substrate amount (kinetic profile)
Buckin & Craig (2005)	Protease	HR-US: Determination of Proteinase K in BSA, in casein aggregates (from bovine milk, 1 % (w/w)) and in pentapeptide (synthetic), 20 °C, determination of ultrasonic velocity and attenuation at four frequencies (real-time)	Increase of ultrasonic velocity over time during enzyme reaction in all investigated substrates, different decrease of ultrasonic attenuation depending on the used frequency; Highest frequency (15 MHz) led to most intense change in attenuation
Born *et al.* (2009)	Protease	URT, determination of different proteases activities (subtilisin, trypsin, halophilic protease from *Haloferax mediterranei* and alkaline protease from *Bacillus lentus*) in casein (0.65 % (w/v)), 25 °C, determination of ultrasonic velocity after activation against blank; Comparison to UV/ Vis/ ninhydrin assay with respect to precision and other analytical parameters for determination of each enzyme	Precisions (10 independent experiments): URT: 5.3 % - 30 % UV/Vis: 1.5 - 4.6 % Low precisions of the URT method were explained by high salt concentrations; Limit of detection/ quantification, sensitivity: both methods similar
Tf-Instruments GmbH (company information)	Amylase, Lipase, Protease	URT: Determination of ultrasonic velocity (real-time) - Determination of amylase activity in starch (0.5 % (w/v)), 25 °C - Determination of lipase activity in not specified olive oil preparation, 37 °C - Determination of protease activity in casein (0.65 % (w/v)), 37 °C, comparison to colorimetric assay	Linear relationship between ultrasonic velocity and enzyme concentrations (Information about enzyme source and procedure are not referenced)
Van Dijk *et al.* (2007)	Amylase, Lipase, Protease and others	HR-US: patented screening method, unspecific and complicated application matrices (e.g. cheese curd), measurement of ultrasonic velocity after incubation	Either increase of ultrasonic velocity or shift of ultrasonic velocity (no kinetics)

Figure 6 demonstrates schematically the assumed change of ultrasonic velocity during an enzymatic hydrolysis reaction. As the compressibility of molecules bound in hydration shells is smaller than in unbound water, ultrasonic velocity depends on the extension of hydration shells in the system. The binding of a protein to a ligand initially causes a release of hydration water and thereby a decrease of ultrasonic velocity (Buckin *et al.*, 2003). Due to hydrolysis of the substrate, the hydration shell expands again and the ultrasonic velocity increases. Both effects interfere with each other leading mostly to a total increase of ultrasonic velocity during hydrolyis (Buckin & Kudryashov, 2002). The feasibility of ultrasonic measurements for determination of

enzyme activity has been widely discussed in the literature. Table 4 summarizes references concerning the ultrasonic determination of amylase, lipase and protease activities.

The ultrasonic detection of starch degradation (5 % in water, pH 6.8) by α-amylase has been previously described (Povey & Rosenthal, 1984). Using the ultrasonic pulse-echo technique, the authors observed a linear dependency of ultrasonic attenuation on amylase activity due to the reduction in viscosity. Ultrasonic velocity was unaffected by amylase activity in their experiment, supposably due to low resolution of the pulsed technique (Povey & Rosenthal, 1984). Using a high resolution device (HR-US), Kudryashov *et al.* (2003) demonstrated that amylolytic activity can also be followed by measuring the ultrasonic velocity.

For enzymatic proteolysis, Buckin & Craig (2005) investigated the use of HR-US. Ultrasonic velocity increased over time as intrinsic compressibility was lost and hydration increased during degradation of three tested substrates. Born *et al.* (2009) determined the degradation of casein by different proteases using URT. The reactions were carried out mostly at pH 8.0, 37°C and stopped by additon of TCA based on the pharmacopoeias. Precipitated proteins were separated. The supernatant was used for ultrasonic measurements at 25°C measured as difference to a blank. The blank was prepared by adding TCA before the addition of the enzyme solution. With respect to precision and other analytical parameters, URT was compared to the UV/ Vis/ ninhydrin assay. The URT method was up to sixfold less precise than the chromogenic assay, possibly due to high high salt concentrations. This can cause high background signals due to large hydration shells around the ions interfering with the signal of casein hydrolysis (Born *et al.*, 2009).

The determination of lipase activity using ultrasonic measurements is rarely discussed in the literature. According to TF Instruments GmbH (company information), the activity of lipase in a non-specified olive oil preparation was determined. A linear relationship between ultrasonic velocity and enzyme concentration was observed for each enzyme. However, no information about enzyme source and procedure were given. Van Dijk *et al.* (2007) patented a method for screening enzymes using HR-US. Matrices like milk, cheese curd or wheat flour suspension allowed the screening of lipase, amylase, protease, oxidase and xylanase activity in the same matrix (US 0264670). Due to non-specificity of the substrates, the described method is not suitable for determining activity of a single enzyme in an enzyme mixture such as Pancreas Powder. The charts provided in the paper to prove the method should be considered critically. The authors observed a difference in ultrasonic velocity between substrate solution and substrate/ enzyme mixture, but an enzyme kinetic curve were often not monitored. Differences in ultrasonic velocity can be caused by an enzyme reaction, but also by the enzyme itself or the enzyme matrix. Therefore, no reliable information about the enzyme reaction can be derived.

Most investigations reviewed in the previous paragraphs were of qualitative nature. Using the Through Transmission Technique, Resa *et al.* (2009) followed a more quantitative approach by developing an ultrasonic velocity assay of extracellular invertase in living yeasts. The authors succesfully determined the effects of substrate concentration (Michaelis-Menten approach),

temperature, pH and enzyme concentration on the increase of ultrasonic velocity and enzyme activity, respectively. Using HR-US, Resa & Buckin (2011) investigated the characteristics of β-glucosidase (cellobiase) which hydrolyzes the O-glycosyl bond of cellobiose whereupon two glucose molecules are released. The authors focused on the "translation" of ultrasonic reaction profiles into reaction progress curves (evolution of concentrations of reactants and products with time) and reaction rate profiles. They calculated the specific concentration increments of ultrasonic velocity of glucose (product), a_P, and cellobiose (substrate), a_S, obtained from the measured values of ultrasonic velocity in aqueous solutions of these compounds as follows:

$$a_i \equiv \frac{v_i - v_0}{v_0 w_i}$$

a_i Specific concentration increment of component *i*

w_i Concentration of component *i*

$v_{0/i}$ Ultrasonic velocity in pure solvent (v_0) and in the solution of component *i* (v_i)

Ultrasonic velocity can be presented as a sum of contributions of its components (Resa & Buckin, 2011):

$$v = v_0 + v_0 \sum_i a_i w_i$$

v_0 Ultrasonic velocity in pure solvent

a_i Specific concentration increment of ultrasonic velocity of component *i*

w_i Concentration (mass fraction) of component *i*

In order to describe the change in ultrasonic velocity in a hydrolysis reaction, the values of a_i and w_i represent the specific concentration increments of ultrasonic velocity and concentrations of substrate, product and solvent, whereupon the concentrations of reactants are linked with each other. Thus, the concentration increment of ultrasonic velocity of the reaction Δa is given by (Resa & Buckin, 2011):

$$\Delta a = a_p \frac{2M_p}{M_s} - a_w \frac{M_W}{M_s} - a_s$$

v_0 Ultrasonic velocity in pure solvent

$a_{p/w/s}$ Specific concentration increment of product (a_p), solvent (a_w) and substrate (a_s)

$M_{p/w/s}$ Molar mass of product (M_p), solvent (M_w) and substrate (M_s)

1.4.5 Ultrasonic determination of emulsion stability

In order to determine lipase activity, a substrate emulsion is needed allowing lipases to adsorb to the oil/ water interface. Instabilities of emulsions can be caused by four mechanisms (Kutz *et al.*, 2011):

- **Creaming and Sedimentation** are universal in emulsions where oil and aqueous phases significantly differ in densities, resulting in an upward or downward movement of the droplets. The rate of creaming or sedimentation increases with droplet size and is intensified by coalescence. While the degree of dispersion remains constant the process is reversible.
- **Flocculation** refers to the aggregation of droplets that occurs when dropets meet during continuous movement due to a lack of repulsive forces. The process is initially reversible.
- **Coalescence** is a consequence of a strong approximation of the droplets and refers to the unification of two or more droplets to one enlarged droplet. The process is irreversible.
- **Breaking** results from proceeding coalescence and refers to complete phase separation.

Low intensity ultrasound has been repeatedly discussed as a means of analyzing stability of dispersions. Howe *et al.* (1986) described a technique to measure emulsion creaming by monitoring the velocity of ultrasound through an emulsion at different heights and times. A concentration profile of the dispersed phase (6.7 MHz) was obtained for the creaming behaviour of hexadecane-in-water emulsions stabilized by nonionic surfactant in the presence and absence of hydroxyethyl cellulose. Buckin *et al.* (2002) monitored the destabilization of a water-in-oil emulsion with a mean droplet size of 0.5 µm induced by temperature rise using different frequencies in the range from 2-15 MHz. An increase of attenuation at 44°C was attributed to the flocculation of dispersed aqueous droplets induced by heating (Figure 7). The

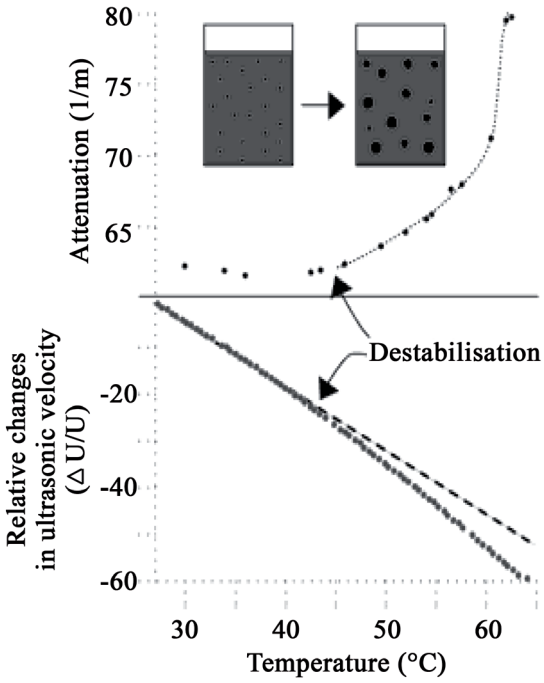

Figure 7. Characterization of the heat stability of a water-in-oil emulsion in a HR-US 102 spectrometer. Ultrasonic velocity and attenuation were monitored at 6 MHz over ascending temperature: The increase of attenuation and the decrease of velocity at 44°C indicate the flocculation of the system (from Buckin *et al.*, 2002)

ultrasonic velocity, which depends on temperature, deviated from the decreasing baseline at the same temperature (44°C). This demonstrates the sensitivity of the ultrasonic parameters to characterize the stability of emulsions. Dukhin *et al.* (2005) studied the use of acoustic spectroscopy to monitor droplet size and fat content in dairy products by measuring ultrasonic attenuation. The authors concluded that attenuation measurements at frequencies below 50 MHz depended on droplet size and thus droplet size can be calculated. A linear correlation between fat content and attenuation was found for frequencies above 50 MHz. The process of coagulation led to a decrease of velocity due to protein aggregation (reduced hydration shells) (Dukhin *et al.*, 2005). Buckin *et al.* (2002) reported the monitoring of sedimentation in a perfluorocarbon emulsion (aqueous, 10 % (v/v)) at 25°C. Sedimentation and coalescence were triggered by the higher density of perfluoro-carbon as compared to water. The sedimentation processes were monitored continuously over 9 h measuring ultrasonic velocity and attenuation in the middle of the cell in the area of the ultrasonic beam. The ultrasonic velocity was used to calculate the volume fraction in the beam, and particle size was calculated from the attenuation (Figure 8). Similar experiments were conducted to investigate the effect of different polymer coatings on the sedimentation rate (Kudryashov *et al.*, 2005). Due to the sensitivity of ultrasonic velocity and attenuation towards emulsion instabilities, the use of a stable substrate emulsion is mandatory for an ultrasonic lipase method to ensure a reliable monitoring of the hydrolyis reaction without including instability processes.

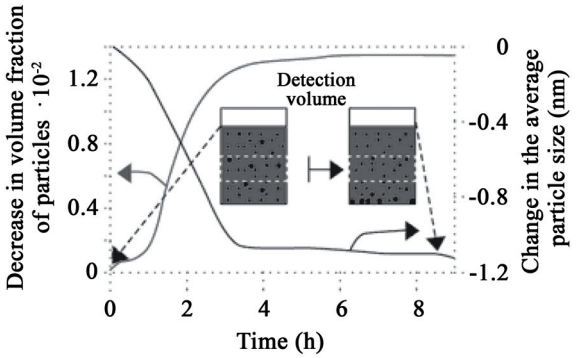

Figure 8. Characterization of the sedimentation of a perfluoro-carbon emulsion in a HR-US 102 spectrometer. Ultrasonic velocity and attenuation were monitored over time in a perfluoro-carbon emulsion to calculate volume fraction in the beam and particle size. Decrease of volume fraction and particle size indicate the sedimentation of perfluoro-carbon (from: Buckin *et al.*, 2002).

1.5 Quality of analytical methods

In order to judge and to ensure quality and reliability of analytical results, method validation is required to confirm that an analytical procedure is suitable for its intended purpose. Many regulatory requirements exist to ensure high standards for analytical methods (ICH Q2 (R1), 1994; U.S. FDA, 2000; CITAC/ Eurachem, 2002). According to ICH, specificity, linearity, range, accuracy, precision and robustness are validation characteristics for assays.

- **Specificity** is the ability to determine the content or potency of an analyte in the presence of components which may be expected to be present (ICH Q2 (R1), 1994; U.S. FDA, 2000). Nearly the same meaning has often been given to selectivity (U.S. FDA, 2001; CITAC/ Eurachem, 2002). In accordance with IUPAC recommendations, specificity is

the ultimate of selectivity. Thus, selectivity can be graded, but a method is either specific or unspecific (Vessman *et al.*, 2001). Both terms will be used in the present thesis, based on the definitions given above.

- **Precision** of an analytical procedure expresses the degree of scatter between several measurements obtained from multiple sampling of the same homogeneous sample. ICH discriminates between three forms with respect to the source of variation: Repeatability (measurement under same operating conditions over a short time interval), intermediate precision (within-laboratory variations such as different days, operators or equipment) and reproducibility (between laboratories) (ICH Q2 (R1), 1994).
- **Accuracy** expresses the closeness of agreement between an accepted reference value (e.g. obtained with pharmacopoeia methods) and the value found (ICH Q2 (R1), 1994).
- **Linearity** of an analytical procedure is its ability to yield results which are directly proportional to the concentration or potency of the analyte. The range of an analytical method is the interval between the upper and lower concentration of analyte for which a suitable precision, accuracy and linearity has been demonstrated (ICH Q2 (R1), 1994).
- **Robustness** shows the reliability of the analysis with respect to deliberate variations in method parameters (ICH Q2 (R1), 1994). Given the relation of ultrasonic velocity to temperature, variation of temperature is one important robustness test within the validation of ultrasonic methods.

1.6 Aim of thesis

The well-known methods for determination of amylase, lipase and protease activity in pancreas powder have persistently remained in the pharmacopoeias, although new methods for enzyme assays have consistently been described in the literature. Based on traditional techniques, the pharmacopoeia methods are optimally adjusted to the demands of pancreas powder analysis and have led to reliable results for many decades. However, they have one essential disadvantage concerning in-process control: They are time-consuming due to multi-step procedures and need extensive sample preparation (e.g. dilution steps or filtering). With the recent development of high-resolution ultrasonic devices, a new technique is available to measure enzyme activity by a simple and fast procedure. Most of the earlier investigations regarding ultrasonic monitoring of enzyme activities are qualitative descriptions. The aim of the present thesis was to evaluate the ultrasonic technique as a tool for the quantitative determination of a single enzyme activity in an enzyme mixture (pancreas powder).

In particular, the feasibility of ultrasonic measurements to determine amylase, lipase, protease and trypsin (single protease) activities was tested and methods were developed that resemble those in the pharamcopoeias with respect to substrates and other conditions. Since ultrasonic

velocity is sensitive to reactions on the molecular level, the focus was on the application of ultrasonic velocity for the determination of pancreatic enzyme activities. In order to evaluate the newly developed methods, the key validation parameters, which have not been described in the literature to date, were determined. Due to the complex nature of in-process samples, various robustness measurements were needed for the following conditions and sample compositions:

- Temperature
- pH of sample matrix
- Salt concentration in sample matrix
- Isopropanol content in sample matrix
- Fat content in sample matrix
- Protein content in sample matrix.

Due to the sensitivity of ultrasonic velocity towards emulsion instabilities, special attention was given to the preparation of a stable substrate formulation for the ultrasonic spectroscopic lipase assay. Applicability of the prepared emulsions was based on measuring stable baselines of ultrasonic velocity. Microscopic analysis and determination of droplet sizes using laser diffraction provided further information. Optimization of the final method were verified by repetition of earlier measurements under newly defined conditions.
While pancreas powder (API) was used for feasibility studies, the final methods were also adopted for pancreas powder-containing products and in-process material. The main focus was on in-process material from early process steps to ensure maximum heterogeneity (worst case scenario). Reference measurements with a working standard were used to correlate with the pharmacopoeia assays, which confirmed the working of the developed assays.
Particular attention was given to investigations on assessing ultrasonic measurements as PAT technique. Besides robustness measurements, time consumption and automation options were examined to determine the feasibility of ultrasonic measurements as in-process control. Finally, the application of ultrasonic measurements for release testing instead of using the traditional pharmacopoeia methods had to be illuminated.

2. MATERIAL AND METHODS

2.1 Materials

2.1.1 Enzymes – Reference standards and samples

For feasibility tests a pancreas powder working standard was used. It was standardized to the official, commercially available FIP and USP standards. The working standard was also applied as reference for the determination of amylase, lipase, protease and trypsin activities in porcine pancreas powder-containing samples to calculate enzyme activity.

Table 5. Amylase, lipase and protease activity of working standard determined according to Ph.Eur.

	Amylase	Lipase	Protease
FIP- Ph.Eur.-u/ g (Working Standard)	93,400	95,200	4,530

The activity label claim of the working standard based on the Ph.Eur. methods described in the monograph 'Pancreas Powder' is shown in Table 5. For trypsin tests, the official FIP standard (42,400 FIP/ Ph.Eur.-u/g) was used.

Table 6. Description of porcine pancreas powder-containing drug products used in the present thesis for feasibility tests of determining total protease activity and for an inter-laboratory test (cps = capsule)

	Drug product I	Drug product II
Application	Feasibility tests of determining total protease activity	Interlaboratory test
Contained porcine pancreas powder in one enteric coated cps (mg)	256	128
Amylolytic activity (USP-u/ cps)	120,000	60,000
Lipolytic activity (USP-u/ cps)	24,000	12,000
Free Protelytic activity (USP-u/ cps)	76,000	38,000
Further components	Cetyl alcohol, triethyl citrate, dimethicone 1000, polyethylene glycol 4000, hypromellose phthalate, gelatine, sodium dodecylsulfate, titanium dioxide, ferric oxide, ferric hydroxide	
Preparation for ultrasonic/ pharmacopoeia measurements	After opening the capsules pellets were pulverized. The pulverized material was used for preparation of the enzyme solutions.	

Porcine pancreas powder-containing product was used for feasibility tests of determining total protease activity due to higher activation potential compared to the working standard (Table 6). Additional drug products were used to determine enzyme activity by ultrasonic measurements to subsequently compare the results with the pharmacopoeia method results (Table 6 and Table 7).

Table 7. Description of porcine pancreas powder-containing enteric coated capsules (cps) used for ultrasonic determination of enzyme activity in drug product in order to compare with pharmacopoeia results

Drug product	III	IV	V	VI	VII	VIII
Porcine pancreas powder contained in one enteric coated capsule (mg)	300	400	128	64	256	32
Amylolytic activity	18,000 Ph.Eur.-u/ cps	25,000 Ph.Eur.-u/ cps	60,000 USP-u/ cps	30,000 USP-u/ cps	120,000 USP-u/ cps	15,000 USP-u/ cps
Lipolytic activity	25,000 Ph.Eur.-u/ cps	40,000 Ph.Eur.-u/ cps	12,000 USP-u/ cps	6,000 USP-u/ cps	24,000 USP-u/ cps	3,000 USP-u/ cps
Protelytic activity Ph.Eur: total protease USP: free protease	1,000 Ph.Eur.-u/ cps	1,600 Ph.Eur.-u/ cps	38,000 USP-u/ cps	19,000 USP-u/ cps	76,000 USP-u/ cps	9,500 USP-u/ cps
Further components	Cetyl alcohol, triethyl citrate, dimethicone 1000, polyethylene glycol 4000, hypromellose phthalate, gelatine, sodium dodecylsulfate, titanium dioxide, ferric oxide, ferric hydroxide					
Preparation for ultrasonic and pharmacopoeia measurements	After opening the capsules pellets were pulverized. The pulverized material was used for preparation of the enzyme solutions.					

Drug substance (API) was determined using material from the end of the manufacturing process of pancreas powder, in composition and appearance comparable to working standard. In-process samples from the beginning of the manufacturing process of pancreas powder were determined:

- Proteolysis material I, three batches
- Proteolysis material II, three batches
- Proteolysis-like material, which is similar to proteolysis material in consistency and appearance, but differs in salt concentration, isopropanol content and enzyme composition (e.g. enhanced trypsin activity).

All in-process samples were liquid aqueous material, containing isopropanol, several salts, fats, proteins and fibers. The in-process material was either frozen immediately after sampling or freshly used on the day of sampling (stored on ice). Prior to ultrasonic measurements, frozen samples were unfrozen. Fibers of highly fibred samples were partially separated using a crude sieve continuing with the ‘filtrates’. The samples were weighed and diluted to 5.0, 10.0 or

20.0 ml with the respective sample solvent (volumetric flask). Prior to the transfer into a beaker the suspension was shaken shortly. The sample solution was stirred for approx. 10 minutes on ice.

2.1.2 List of reagents

- N-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) (Calbiochem/ Merck, Darmstadt, Germany)
- Boric acid p.a. (Riedel-de Haën/ sigma aldrich, Seelze, Germany)
- Calcium chloride dihydrate p.a. (Merck, Darmstadt, Germany)
- Casein Bovine Milk (Calbiochem/ Merck, Darmstadt, Germany)
- Dextrin 5/ 10/ 20 from maize starch (Fluka/ sigma aldrich, Buchs, Switzerland)
- Enterokinase, 0.5 u/ 1 mg (FIP, Int. Commission on Pharmaceutical Enzymes, Centre of standards, Gent, Belgium)
- Ethanol absolute (Mallinckrodt Baker B.V., Deventer, Netherlands)
- Glyceryl trioctanoate min. 99% (sigma-aldrich, Steinheim, Germany)
- Gum arabic spray-dried, Ph. Eur. (Merck, Darmstadt, Germany)
- Hydrochloric acid 1M (Merck, Darmstadt, Germany)
- Iodine solution 0.05M Titripur® (Merck, Darmstadt, Germany)
- Lipase activating mixture Ph.Eur. (FIP/ Ph.Eur.) [“Galle dispert”]
- Maleic anhydride, pure (Fluka/ sigma aldrich, Buchs, Switzerland)
- Olive oil (Roth, Karlsruhe, Germany)
- Pancreatic amylase (56,300 u/g), lipase (41,400 u/g) and protease (1,480 u/g) (FIP, Int. Commission on Pharmaceutical Enzymes, Centre of standards, Gent, Belgium)
- Pancreatin amylase and protease/ Pancreatin lipase (USP, Basel, Switzerland)
- Potassium phosphate monobasic p.a. (sigma aldrich, Seelze, Germany)
- 2- Propanol 99 % (Merck, Darmstadt, Germany)
- Sodium chloride p.a. (Merck, Darmstadt, Germany)
- di-Sodium hydrogen phosphate anhydrous p.a. (Merck, Darmstadt, Germany)
- di-Sodium hydrogen phosphate dihydrate p.a. (Merck, Darmstadt, Germany)

- Sodium hydroxide solution 1M Titripur® (Merck, Darmstadt, Germany)
- Sodium hydroxide solution 0.02M Titripur® (Merck, Darmstadt, Germany)
- Sodium thiosulfate solution 0.1M Titripur® (Merck, Darmstadt, Germany)
- di-Sodium tetraborate decahydrate p.a. (Riedel-de Haën/ sigma aldrich, Seelze, Germany)
- Starch, soluble p.a. (Merck, Darmstadt, Germany)
- Sulfuric acid 95-97 % (sigma aldrich, Seelze, Germany)
- Titrisol® concentrate for sodium hydroxide solution 0.1 mol/l (Merck, Darmstadt, Germany)
- Trichloroacetic acid (TCA) p.a. (sigma Aldrich, Seelze, Germany)
- Tris(hydroxymethyl) aminomethane (TRIS) p.a. (Merck, Darmstadt, Germany)
- Triton X 100, pure (Roth, Karlsruhe, Germany)
- Trypsin, 42,400 u/g (FIP, Int. Commission on Pharmaceutical Enzymes, Centre of standards, Gent, Belgium)
- Working standard (according to 2.1.1)

Deionized water was used (resistance > 0.18 MΩ·m, organic carbon < 0.5 mg/l).

2.1.3 Preparation of buffers and solutions

2.1.3.1 Reagents for determination of amylase activity

Amylase solution (URT/HR-US)

Working standard (20 mg) or a porcine pancreas powder containing sample containing approx. 1870 FIP/ Ph.Eur.-units, respectively), and accurately weighed, were dissolved in 40.0 ml Phosphate buffer solution (URT/ HR-US) for approx. 15 min in ice.

Amylase solution (URT/HR-US)

Working standard (20 mg) or a porcine pancreas powder containing sample containing approx. 1870 FIP/ Ph.Eur.-units, respectively), and accurately weighed, were dissolved in 40.0 ml Phosphate buffer solution 0.2 M (URT/ HR-US) for approx. 15 min in ice.

Phosphate buffer solution (URT/ HR-US)

100 ml of phosphate buffer 0.2 M (Ph.Eur./ USP) pH 6.8 and 33.5 ml of sodium chloride solution 0.2 M (Ph.Eur./ USP) were diluted to 1000 ml water.

Phosphate buffer solution 0.2 M (URT/ HR-US)

392 mg of sodium chloride were dissolved in 1000 ml Phosphate buffer 0.2 M pH 6.8 (Ph.Eur/ USP).

Starch solution 6 %/ 3 % (w/v) (URT/ HR-US)

A portion of starch, equivalent to 3.0/ 6.0 g of dried substance, was grinded with approx. 10 ml Phosphate buffer solution (URT/ HR-US). The mixture was added to approx. 70 ml boiling Phosphate buffer solution (URT/ HR-US), stirring on a heating plate (Hot plate RCT basic, IKA Werke GmbH & Co. KG, Staufen, Germany). The starch solution was boiled again and was cooled down to room temperature subsequently. The solution was diluted to 100.0 ml with Phosphate buffer solution (URT, HR-US). Higher volumes of starch solution were heated by an immersion heater (e.g. Rommelsbacher Elektro-Hausgeräte GmbH, Dinkelsbühl, Germany).

Amylase solution (Ph.Eur./ USP)

A quantity of substance, equivalent to about 1500 Ph.Eur-units (or 20 mg of USP Pancreatin Amylase and Protease according USP or quantity equivalent to this activity), accurately weighed, were triturated with 60 ml (30 ml according USP) of Phosphate buffer 0.2 M pH 6.8 (Ph.Eur/ USP) for 15 min. The solution was transferred to a volumetric flask and diluted to 100.0 ml (50.0 ml according USP) with Phosphate buffer 0.2 M pH 6.8 (Ph.Eur./ USP).

Phosphate buffer 0.2 M pH 6.8 (Ph.Eur/ USP)

Approx. 49 ml of di-sodium hydrogen phosphate dehydrate solution (35.6 g/l, Ph.Eur.) or approx. 49 ml of anhydrous dibasic sodium phosphate solution (28.4 g/l, USP) were added to 51 ml of monobasic potassium phosphate solution (27.2 g/l). The solution was stirred and the pH was controlled until pH 6.8 was achieved.

Sodium chloride solution 0.2 M (Ph.Eur./ USP)

Sodium chloride (11.7 g) was dissolved to 1000 ml water.

Starch solution 1 % (w/v) (Ph.Eur./ USP)

A portion of starch, equivalent to 10.0 g of dried substance, was grinded with approx. 10 ml water. This mixture was added to approx. 700 ml boiling water (Immersion heater, e.g. Rommelsbacher Elektro-Hausgeräte GmbH, Dinkelsbühl, Germany). The beaker was rinsed with water and the washings were added to the hot solution. The starch solution was boiled again and was cooled down to room temperature subsequently. The solution was diluted to 1000.0 ml with water.

2.1.3.2 Reagents for determination of lipase activity

Bile salts solution (URT/ HR-US)

"Galle dispert" (lipase activating mixture Ph.Eur.) (1263 mg) was dissolved in 100.0 ml lipase buffer.

Maleate buffer solution pH 7.0 (URT/ HR-US)

Sodium chloride (10.0 g), TRIS (6.06 g) and maleic anhydride (4.90 g) were dissolved in 900 ml water. The pH was adjusted with sodium hydroxide solution to pH 7.0. The solution was diluted to 1000.0 ml with water (equivalent to Ph.Eur.).

Lipase solution (URT/ HR-US)

Working standard (15 mg) or a porcine pancreatic powder containing sample containing approx. 1428 FIP/ Ph.Eur.-units, respectively, and accurately weighed, were dissolved in 5.0 ml Maleate buffer solution pH 7.0 (URT/ HR-US) for approx. 15 min in ice.

Olive oil emulsion (URT/ HR-US) (used for initial experiments)

Triton X 100 (780 µl) was dissolved in approx. 220 ml of TRIS buffer solution (URT/ HR-US) for determination of Trypsin activity. Olive oil (15 ml) was added and diluted to 250.0 ml with TRIS buffer solution (URT/ HR-US) for determination of Typsin activity. This dispersion was transferred into a blender (BL 450, Kenwood Limited, Havant, United Kingdom) and mixed at the first grade for 15 min. After one to two hours the pre emulsion was processed using a Microfluidizer® 110-EH Processor (Microfluidics, Lampertheim, Germany) with cooling at 1200 bar and three passages. Interaction chambers F12Y upstream and H30Z downstream were used. This emulsion (25 ml) was diluted with 25 ml of Reagents mixture (Ph.Eur./ USP).

Olive oil emulsion (URT/ HR-US) (final emulsion)

Triton X 100 (2.0 ml) was dissolved in approx. 220 ml of TRIS buffer solution (URT/ HR-US) for determination of Lipase activity. Olive oil (15 ml) was added and diluted to 250.0 ml with TRIS buffer solution (URT/ HR-US) for determination of Lipase activity. This dispersion was transferred into a blender (BL 450, Kenwood Limited, Havant, United Kingdom) and mixed at the first grade for 15 min. After one to two hours the pre emulsion was processed using a Microfluidizer® 110-EH Processor (Microfluidics, Lampertheim, Germany) with cooling at 1000 bar and four passages. Interaction chambers F12Y upstream and H30Z downstream were used. This emulsion (25 ml) was diluted with 25 ml of bile salts solution.

TRIS buffer solution (URT/ HR-US) for determination of lipase activity

Calcium chloride dihydrate (17.64 g) and TRIS (73.2 g) were dissolved in approx. 1800 ml water. The pH was adjusted to 7.2 with hydrochloric acid at 37 °C. The solution was diluted to 2000.0 ml with water at room temperature.

Maleate buffer solution pH 7.0 (Ph.Eur.)

Sodium chloride (10.0 g), TRIS (6.06 g) and maleic anhydride (4.90 g) were dissolved in 900 ml water. The pH was adjusted with sodium hydroxide solution to pH 7.0. The solution was diluted to 1000.0 ml with water (equivalent to URT/ HR-US).

Lipase solution (Ph.Eur/ USP)

According to Ph. Eur. a quantity of substance, equivalent to about 2500 Ph.Eur-units, accurately weighed, were triturated with 1 ml of cooled maleate buffer solution pH 7.0 and diluted with cold maleate buffer solution pH 7.0 stirring for 15 min. The solution was qualitatively transferred to a volumetric flask and diluted to 100.0 ml. According to USP Lipase solution using USP Pancreatin Lipase or a quantity equivalent to this activity, was prepared as described above, but water was used as solvent and the volume was measured in a measuring cylinder.

Olive oil stock emulsion (Ph.Eur./ USP)

Olive oil (40 ml), 10 % (w/v) acacia solution (330 ml) and water (ice, 30 ml) were filled in the cup on an electric blender (BL 450, Kenwood Limited, Havant, United Kingdom) and were mixed for 15 min at high speed.

Reagents mixture (Ph.Eur./ USP)

“Galle dispert” (Lipase activating mixture Ph.Eur.) (100 ml) or USP Bile salts solution (8 % (w/v), 450 ml water and 400 ml of a 5 mM TRIS solution, containing 40 mM sodium chloride, were mixed.

2.1.3.3 Reagents for determination of free protease activity

Borate buffer pH 7.5 (URT/ HR-US)

Sodium chloride (12.5 g), di-sodium tetraborate decahydrate (14.25 g) and boric acid (52.5 g) were dissolved in approx. 4500 ml water. The pH was adjusted to 7.5 with hydrochloric acid or sodium hydroxide solution. The solution was diluted to 5000.0 ml with water.

Casein solution 1.25 % pH 8.0 (URT/ HR-US)

Casein, equivalent to 1.25 g of dried substance was suspended in 5 ml of water. Sodium hydroxide solution 0.1 M (10 ml) was added and stirred for 1 min. Water (60 ml) was added and stirred with a magnetic stirrer until the solution was practically clear (approx. 1 h). The pH was adjusted to 8.0 with 0.1 M sodium hydroxide or 0.1 M hydrochloric acid. The solution was diluted to 100.0 ml with water (equivalent to USP). For initial experiments, a 0.625 % Casein solution was prepared in the same way using 0.625 g casein.

Free protease solution (URT/ HR-US)

Working standard (25 mg) or a porcine pancreatic powder containing sample containing approx. 113 FIP/ Ph.Eur.-units, respectively, were dissolved in 5.0 ml of Borate buffer pH 7.5 (URT/ HR-US) for approx. 15 min in ice.

Casein solution 1.25 % pH 8.0 (USP)

Casein, equivalent to 1.25 g of dried substance was suspended in 5 ml of water. Sodium hydroxide solution 0.1 M (10 ml) was added and stirred for 1 min. Water (60 ml) was added and stirred with a magnetic stirrer until the solution was practically clear (approx. 1 h). The pH was adjusted to 8.0 with 0.1 M sodium hydroxide or 0.1 M hydrochloric acid. The solution was diluted to 100.0 ml with water (equivalent to URT/ HR-US).

Free protease solution (USP)

A quantity of USP Pancreatin Amylase and Protease or substance, which conformed to an end concentration of 2.5 USP-units per milliliter in the diluted solution, accurately weighed, was added to 100.0 ml of Phosphate buffer solution (USP) and stirred in ice. This solution (2.0 ml) was diluted in a volumetric flask with phosphate buffer solution pH 7.5 to 100.0 ml.

Phosphate buffer solution (USP)

Monobasic potassium phosphate (6.8 g) and sodium hydroxide (1.8 g) were dissolved in 950 ml water in a 1000 ml volumetric flask. The pH was adjusted to 7.5 ± 0.2, using 0.2 M sodium hydroxide. The volume was adjusted with water.

Trichloroacetic acid solution (USP)

Trichloroacetic acid (50 g) was dissolved in 1000 ml of water.

2.1.3.4 Reagents for determination of total protease activity

Calcium chloride solution 0.02 M (URT/ HR-US)

Calcium chloride dihydrate (14.7 g) was dissolved in water (approx. 4500 ml). The pH was adjusted to 6.1 ± 0.1 by hydrochloric acid or sodium hydroxide solution. The solution was diluted to 5000.0 ml with water.

Casein solution 1.25 % pH 8.0 (URT/ HR-US)

Casein, equivalent to 1.25 g of dried substance was suspended in 5 ml of water. Sodium hydroxide solution 0.1 M (10 ml) was added and stirred for 1 min. Water (60 ml) was added and stirred with a magnetic stirrer until the solution was practically clear (approx. 1 h). The pH was adjusted to 8.0 with 0.1 M sodium hydroxide or 0.1 M hydrochloric acid. The solution was diluted to 100.0 ml with water (equivalent to Ph.Eur.).

Enterokinase solution (URT/HR-US)

Enterokinase (330 mg) was dissolved in 20.0 ml of a 0.02 M Calcium chloride solution (URT/ HR-US) in an ice bath. The solution was stored in ice.

Total protease solution (URT/ HR-US)

As reference 25 mg of working standard were dissolved in 10.0 ml of 0.02 M Calcium chloride solution (URT/ HR-US) for approx. 15 min in ice.
In order to activate the pro enzymes, a substance amount containing approx. 280 (activated) FIP/ Ph.Eur.-units was dissolved in 5.0 ml of a 0.02 M Calcium chloride solution (URT/ HR-US). Ice-cold enterokinase solution (2.0 ml) was tempered for 5 min at 35 °C ± 1°C. After adding 0.5 ml of the ice-cold enzyme solution, the sample was activated for 15 min at 35 °C ± 1 °C. Subsequently, the activated sample was stored in ice.

Borate buffer pH 7.5 (Ph.Eur.)

Sodium chloride (12.5 g), di-sodium tetraborate decahydrate (14.25 g) and boric acid (52.5 g) were dissolved in approx. 4500 ml water. The pH was adjusted to 7.5 with hydrochloric acid or sodium hydroxide solution. The solution was diluted to 5000.0 ml with water.

Calcium chloride solution 0.02 M (Ph.Eur.)

Calcium chloride dihydrate (14.7 g) was dissolved in water (approx. 4500 ml). The pH was adjusted to 6.1 ± 0.1 by hydrochloric acid or sodium hydroxide solution. The solution was diluted to 5000.0 ml with water.

Casein solution 1.25 % pH 8.0 (Ph.Eur.)

Casein, equivalent to 1.25 g of dried substance was suspended in 5 ml of water. Sodium hydroxide solution 0.1 M (10 ml) was added and stirred for 1 min. Water (60 ml) was added and stirred with a magnetic stirrer until the solution was practically clear (approx. 1 h). The pH was adjusted to 8.0 with 0.1 M sodium hydroxide or 0.1 M hydrochloric acid. The solution was diluted to 100.0 ml with water (equivalent to URT/ HR-US).

Enterokinase solution (Ph.Eur.)

Enterokinase (50 mg) were dissolved in Calcium chloride solution 0.02 M (Ph.Eur.). The solution was diluted to 50.0 ml with the same solvent.

Total protease solution (Ph.Eur.)

As reference a substance amount, containing approx. 130 Ph.Eur.-units, were triturated in approx. 90 ml of cooled Calcium chloride solution 0.02 M (Ph.Eur.). After adjusting the pH to 6.1 ± 0.1 the solution was quantitatively transferred into a volumetric flask and diluted to 100.0 ml with Calcium chloride solution 0.02 M (Ph.Eur.). This solution (5.0 ml) was diluted with Borate

buffer pH 7.0 (Ph.Eur.) to 100.0 ml. To activate the pro enzymes, a substance amount containing approx. 260 Ph.Eur.-units was triturated in approx. 90 ml of cooled Calcium chloride solution 0.02 M (Ph.Eur.). After adjusting the pH to 6.1 ± 0.1 the solution was quantitatively transferred into a volumetric flask and diluted to 100.0 ml with calcium chloride solution 0.02 M (Ph.Eur.). Enterokinase solution (Ph.Eur.) (5.0 ml) was tempered to 35°C ± 1°C. After adding 5.0 ml of the ice cold enzyme solution and subsequently mixing the sample was activated for 15 min at 35°C ± 1°C. Afterwards the activated sample was cooled down in ice. This solution (5.0 ml) was diluted with Borate buffer pH 7.0 (Ph.Eur.) to 100.0 ml.

Trichloroacetic acid solution (Ph.Eur.)
Trichloroacetic acid (50 g) was dissolved in 1000 ml of water.

2.1.3.5 Reagents for determination of free trypsin activity

Hydrochloric acid 1 mM (URT/ HR-US)
Hydrochloric acid 1 M (1 ml) was dissolved to 1000.0 ml with water.

Triton X 100 solution (URT/ HR-US)
Triton X 100 (0.6 mM) was diluted in 1000 ml Hydrochloric acid 1 mM (URT/ HR-US).

TRIS buffer (URT/ HR-US) for determination of Trypsin activity
TRIS (12.2 g) and calcium chloride dihydrate (2.94 g) were dissolved in 800 ml water. The pH was adjusted to 7.8 at 37 °C. The solution was diluted to 1000.0 ml with water.

BAEE solution (URT/HR-US)
BAEE (20 mg) was dissolved to 10.0 ml with TRIS buffer (URT/ HR-US) for determination of Trypsin activity.

Trypsin solution (URT/ HR-US)
Working standard (50 mg) or a porcine pancreas powder containing sample or containing approx. 65 FIP/ Ph.Eur.-units or 1.5 mg of FIP Trypsin Reference Standard, respectively, was dissolved in 5.0 ml of Triton X 100 solution (URT/ HR-US) for approx. 15 min in ice.

Borate buffer 0.0015 M pH 8.0 (Ph.Eur.)
Borax (572 mg) and calcium chloride dihydrate (2.94 mg) were dissolved in 900 ml water. After adjusting the pH to 8.0 the solution was diluted to 1000.0 ml with water.

Calcium chloride solution 0.02 M (Ph.Eur.)
Calcium chloride dihydrate (14.7 g) was dissolved in approx. 4500 ml water. The pH was

adjusted to 6.1 ± 0.1 by hydrochloric acid or sodium hydroxide solution and diluted to 5000.0 ml with water.

BAEE solution 0.02 M (Ph.Eur.)
BAEE (171.3 mg) were dissolved in 25 ml water.

Trypsin solution (Ph.Eur.)
FIP Trypsin Reference Standard (12 mg) or a porcine pancreas powder containing sample containing approx. 509 FIP/ Ph.Eur.-units, respectively, was dissolved in 50.0 ml of Calcium chloride solution 0.02 M (Ph.Eur.).

2.2 Methods

2.2.1 Ultrasonic measurements

2.2.1.1 General

Ultrasonic measurements were performed with a ResoScan® Research System from TF Instruments GmbH (Heidelberg, Germany) using Ultrasonic Resonator Technology (URT) and with a HR-US 102 P spectrometer from Sonas Technologies Ltd. (Dublin, Ireland). Both are high resolution devices based on resonance (build-in systems). Considering automation, a Flow-Through System HR-US EX-FTC (Sonas Technologies Ltd., Dublin, Ireland) was tested.

2.2.1.2 Ultrasonic measurements using the ResoScan Research System (URT)

The ResoScan® Research System is equipped with two closed sample cells with a path length of 7.0 mm and a volume of approx. 200 ml. Evaporation of the sample is prevented by gas-tight lids. In the cells, the samples are tempered with a rate of 0.10 to 0.35 °C per min via Peltier elements. The absolute accuracy of the thermostat temperature is 0.01 °C. The resolution of the ultrasonic velocity is 0.001 m/s and the resolution of ultrasonic attenuation is 1-3 %, depending on the extent of attenuation.

Table 8. URT initialization settings for determination of pancreatic enzyme activity. Italics in brackets indicate the

Enzyme	Substrate preparation	Peak order number	Power (dB)
Amylase	Starch solution 1 % (Ph.Eur./ USP)	75 *(approx. 8.2 MHz)*	0
	Starch solution 3 %/ 6 % (URT/ HR-US)	75 *(approx. 8.2 MHz)*	6
Lipase	Olive oil emulsion (URT/ HR-US, final emulsion)	75 *(approx. 8.3 MHz)*	6
Protease	Casein solution 0.625 %/ 1.25 % (URT/ HR-US)	74 *(approx. 8.1 MHz)*	0
Trypsin	BAEE solution (USP/ HR-US)	70 *(approx. 7.7 MHz)*	0

Prior to measurement a master peak is defined via initialization. The master peak is used for calculation of the ultrasonic parameters. For this, the frequency range of about 7 to 9 MHz is scanned obtaining a series of resonance peaks. The software evaluates the series of resonance peaks concerning deviation from real resonator frequencies and amplitude. The master peak can be defined prior to initialization by entering the peak order number.
To achieve sufficient amplitudes of the resonance peaks the applied voltage input needs to be adapted depending on the sample properties. The amplitude is controlled by varying the power. Used settings of initialization are listed in Table 8.

Determination of enzyme kinetic was performed as follows:

The operating temperature was 37°C. Both cells were filled with 180 µl tempered substrate solution using a Multipette stream (Eppendorf AG, Hamburg, Germany) and closed. The difference in ultrasonic velocity between both cells was measured until steady state (baseline check). One of the cells was opened again to inject 4 µl of either amylase, lipase or free protease solution or 8 µl of either trypsin or total protease solution using a Multipette stream or pipette research (Eppendorf AG, Hamburg, Germany). The content in the cell was mixed carefully to avoid creating air bubbles with a pipette reference (Eppendorf AG, Hamburg, Germany). After closing the cell, the ultrasonic measurement was started. The change of ultrasonic velocity (m/s) over time (s) was determined twice for each enzyme solution.

Alternatively, the sample cell was filled by using the pre-mix method:
1800 µl tempered substrate solution and 40µl of either amylase solution, lipase solution or free protease solution or 80 µl of either trypsin solution or total protease solution were pre-mixed (gentle shaking for 5 sec). The sample cell was filled with 184 µl or 188 µl of this pre-mix using a Multipette stream (Eppendorf AG, Hamburg, Germany).

ResoPump I RCU (Tf Instruments GmbH, Heidelberg, Germany) was used for cell draining. After each measurement, cleaning and pre-conditioning were carried out by rinsing the system as follows:

1. Tempered low concentrated surfactant (commercial dish liquid) solution for approx. 10 sec
2. Tempered pure water for approx. 10 sec
3. 180 µl tempered substrate solution (substrate refilling)

Ultrasonic velocity ($m \cdot s^{-1}$) was plotted over time (s). Due to temperature instabilities the first data points after injection (40 sec) were excluded. The data points of the next 200 sec were

used to calculate the slope ($m \cdot s^{-2}$) per linear regression. The arithmetic mean and the standard deviation of the two replicates were calculated. The slope was used to determine enzyme activity based on the slope obtained with reference standards (see 2.2.1.7).

2.2.1.3 Ultrasonic measurements using the HR-US 102 P spectrometer

The HR-US 102 P spectrometer is equipped with two closed sample cells with a volume of approx. 1500 µl. In the cells, the samples are tempered via external programmable water bath C25P with drop-in circulator phoenix II P1 (Thermo Scientific GmbH, Karlsruhe). The absolute accuracy of the thermostat temperature is 0.01 °C. The differential regime allows a resolution of 0.0002 m/ s for the ultrasonic velocity and 0.2 % for ultrasonic attenuation.
Prior to measurement, a peak scan is performed to define the master peak, which is used for calculation of the ultrasonic parameters. Therefore a defined frequency range (between 2 and 15 MHz) is scanned obtaining a series of resonance peaks. One peak is chosen to transfer the peak settings to the system for the following measurements.
To achieve sufficient amplitudes of the resonance peaks the applied voltage input needs to be adapted depending on the sample properties. The amplitude is controlled by varying the input voltage. Used settings of the peak scan are listed in Table 9.

Table 9. HR-US and HR-US FTS peak scan settings for determination of pancreatic enzyme activity.

Enzyme	Substrate preparation	Frequency of master peak (MHz)	Voltage Input (V)
Amylase	Starch solution 3 %/ 6 % (URT/ HR-US)	approx. 8.1	0.4
Lipase	Olive oil emulsion (URT/ HR-US, final emulsion)	approx. 8.2	1.0
Protease	Casein solution 0.625 %/ 1.25 % (URT/ HR-US)	approx. 8.0	0.4
Trypsin	BAEE solution (USP/ HR-US)	approx. 8.0	0.2

Enzyme activity was determined as follows:

The water bath was set to 37°C. Both cells were filled with 1050 µl degassed substrate solution using a Multipette stream (Eppendorf AG, Hamburg, Germany) and closed. The difference in ultrasonic velocity between both cells was measured until steady state (baseline check). One of the cells was opened again to inject 20 µl of either amylase, lipase, protease or trypsin solution using a Multipette stream (Eppendorf, Hamburg, Germany). The content in the cell was mixed carefully to avoid creating air bubbles with a pipette reference (Eppendorf AG, Hamburg, Germany). After closing the cell, the ultrasonic measurement was started. The change of ultrasonic velocity (m/s) over time (s) was determined twice for each enzyme solution.
An injection of the enzyme solution using a syringe through a septum was also possible.

Alternatively, the sample cell was filled by using the pre-mix method: 1800 µl tempered substrate solution and 40µl of either amylase, lipase or free protease solution or 80 µl of either trypsin or total protease solution were pre-mixed (gentle shaking for 5 sec). The sample cell was filled with 1050 µl of this pre-mix using a Multipette stream (Eppendorf AG, Hamburg, Germany). A pump Laboport KT18 for vacuum system (KNF Neuberger AG, Balterswil, Switzerland) was used for cell draining. After each measurement cleaning and pre-conditioning were carried out by rinsing the system as follows:

1. Low concentrated surfactant (commercial dish liquid) solution for approx. 10 sec
2. Pure water for approx. 10 sec
3. Ethanol absolute for drying (few droplets)

Ultrasonic velocity ($m \cdot s^{-1}$) was plotted over time (s). Due to temperature instabilities the first data points after injection (250 sec) were excluded. The data points of the next 200 sec were used to calculate the slope ($m \cdot s^{-2}$) per linear regression. The arithmetic mean and the standard deviation of the two replicates were calculated. The slope was used to determine enzyme activity based on the slope obtained with reference standards (see 2.2.1.7).

2.2.1.4 Ultrasonic measurements using the Flow-Through System HR-US EX-FTS

In order to test automation opportunities of ultrasonic velocity measurements, a Flow-Through System (FTS) was tested. The Flow-Through System HR-US FTS is equipped with two Flow through cells with a cell volume of 1 ml. Figure 9 shows water, substrate and sample flow for the HR-US 102 P and for the FTS in comparison (for the sake of clarity the resonator technique in the instruments is omitted in Figure 9). The systems differ in the operating procedure as explained below.

Working basically with the HR-US 102 P spectrometer, there are two possibilities to fill the cell before measurement:

1. Adding enzyme solution after baseline check
2. Adding substrate and enzyme solution simultaneously after pre-mixing

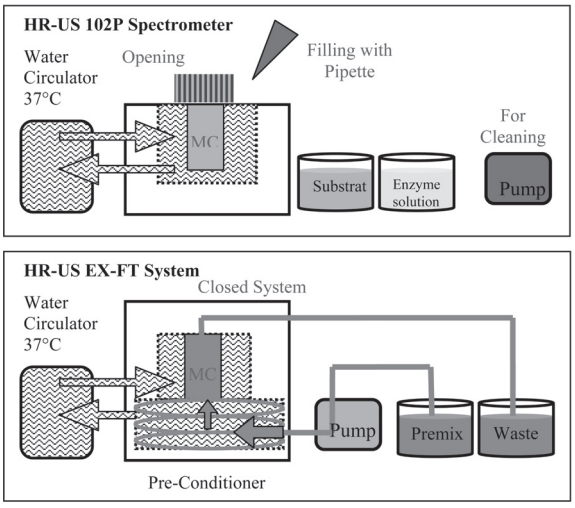

Figure 9. Schematic water, substrate and sample flow for HR-US 102P and HR-US EX-FTS (Resonator technique is omitted). In contrast to the HR-US 102, in the FTS the sample is pre-tempered before reaching the measurement cell (MC). The FTS is a closed system, whereas the cell in the HR-US 102 P has to be opened for filling (Sonas Technologies Ltd.).

Using the FTS allows only the second option (pre-mixing).

For the tubes used at the inlet and outlet as well as the inside of the FTS instrument, the material Tygon® 2275 was chosen. A four-channel microprocessor-controlled dispensing pump (Ismatec Laboratoriumstechnik GmbH, Wertheim-Mondfeld, Germany) served for filling the cells. The pump was operated with two Tygon® 3607 tubes.
The flow rate was adjusted to 13.3 ml/ min (+/- 0.9 %) for starch solution, 13.5 ml/ min (+/- 1.4 %) for casein solution and 11.9 ml/ min (+/- 3.5 %) for olive oil emulsion. One channel was used for filling the reference cell, the other channel for filling the sample cell.
The operating temperature was 37 °C. First both cells were filled with degassed substrate solution (55 sec). Degassed substrate solution (15.75 ml) and 300 µl of either amylase, lipase, protease or trypsin solution were mixed in a sample tube (50 ml) for 45 sec using a pipette reference (Eppendorf AG, Hamburg, Germany). This pre-mix was pumped into the measuring cell (55 sec). The reference cell was rinsed with pure degassed substrate solution with backflow. The ultrasonic measurement was started as soon as the pump stopped. Each enzyme solution was determined twice.

Cleaning was carried out by rinsing the system as follows:

1. Low concentrated surfactant (commercial dish liquid) solution for approx. 30 sec
2. Pure water for approx. 30 sec

Ultrasonic velocity ($m \cdot s^{-1}$) was plotted over time (s). Due to temperature instabilities the first data points after injection (100 sec) were excluded. The data points of the next 200 sec were used to calculate the slope ($m \cdot s^{-2}$) per linear regression. The arithmetic mean and the standard deviation of the two replicates were calculated. The slope was used to determine enzyme activity based on the slope obtained with reference standards (see 2.2.1.7).

2.2.1.5 Ultrasonic methods for determination of amylase, lipase, protease and trypsin activity using URT, HR-US or HR-US EX FTS

The general performance of ultrasonic measurements to determine enzyme activity using URT, HR-US or HR-US EX FTS are described in the preceding chapters (2.2.1.2 - 2.2.1.3). The ultrasonic methods for determination of amylase, lipase, protease and trypsin activity differ in used substrate and enzyme solution. Introduced combinations are summarized in Table 10.

The ultrasonic methods defined in Table 10 were validated. In order to determine **specificity**, ultrasonic velocity change over time was measured in samples to which either substrate or

enzyme had not been added as well as in complete samples (positve control). Two working standard solutions (two replicates each) per case were determined. Additionally, specificity was tested by adding substances (sodium chloride, calcium, isopropanol, protein, fat and emulsifying agents) to the working standard solutions, which were expected to be present in in-process samples. Table 11 shows the used reagents and sample solvent preparation.

Repeatability was determined by measuring six enzyme solutions (two replicates each) the same day by one operator. The standard deviation and CV of the six determinations were calculated. The **intermediate precision** was determined by measuring two enzyme solutions (two replicates each) by three operators at different days. The standard deviation and the CV of the six determinations were calculated. Both tests were performed by using different methods of enzyme addition (Pre-mixing method and cell injection using a pipette (after opening the cells) or a syringe (via septum)). Special repeatability experiments were done with highly concentrated samples or process material.

Table 10. Combinations of substrate solution and enzyme solution used for ultrasonic methods to determine pancreatic amylase, lipase, protease and trypisn activity.

Pancreatic enzyme	Name of ultrasonic method	Substrate solution	Enzyme solution
Amylase	US-A	Starch solution 3 %/ 6 % (URT/ HR-US)	Amylase solution (URT/ HR-US)
Lipase	US-L	Olive oil emulsion (URT/ HR-US)	Lipase solution (URT/ HR-US)
Protease (free)	US-Pf	Casein solution 1.25 % (URT/ HR-US)	Free protease solution (URT/ HR-US)
Protease (total)	US-Pt		Total protease solution (URT/ HR-US)
Trypsin	US-Tf	BAEE solution (URT/ HR-US)	Trypsin solution (URT/ HR-US)

Table 11. Reagents and sample solvent preparation for specificity tests concerning matrix substances which are expected in in-process samples.

Substance in sample matrix tested	Model	Preparation of sample solvent
Sodium chloride	-	Dissolution in the sample solvent, pH-control
Calcium	Calcium chloride	
Isopropanol	-	Substitution of water with isopropanol
Protein	Casein	Dissolution in the sample solvent, pH-control
Fat	Olive oil	Addition of olive oil to the sample solvent and mixing in a blender (BL 450 Kenwood Limited, Havant, United Kingdom) for 10 minutes (grade 2)/ Immediately after mixing dissolution of the working standard in the oily sample solvent
Emulsifying substances/ fat	Triton X 100/ Olive oil	Triton X 100 was dissolved in the sample solvent before addition of olive oil and mixing in a blender (BL 450 Kenwood Limited, Havant, United Kingdom) for 10 minutes (grade 2)/ Immediately after mixing dissolution of the working standard in the oily sample solvent

Table 12. Overview of experimental sequences for the determination of pancreatic enzyme activity in proteolysis-like material, proteolysis material, API and drug products. US-X-URT/HR-US describes the ultrasonic methods according to Table 10 (rep = replicates).

		Proteolysis-like material	Proteolysis material I	Proteolysis material II	API	Drug product II	Drug product III-VIII
Working Standard solutions (URT/ HR-US)		2 (before measurement)	2 (before measurement) (Trypsin = FIP standard)	3 (before measurement)	5 (2 before and 3 after measurement)	2 (before and after measurement)	3 (2 before and 1 after measurement)
Calibration according to 2.2.1.8 (URT-HR-US)		One-point	One-point	Three-point	One-point/ Three-point	One-point	One-point
Separation of fibers (URT/ HR-US)		yes	yes	no	-	-	-
Cell injection/ Premix (URT/ HR-US)		Cell injection	Cell injection	Premix	Cell injection: L, Pf, Premix: A, Pt	Cell injection: A, Pf, Premix: L	Cell injection: A, L, Pf, Premix: A, L, Pf, Pt
Number of experiments (replicates) (URT/ HR-US)		2 (1 rep)	2 (1rep)	2 (2 rep)	2 (2 rep)	6 (2 rep) on two days	2 (2 rep)
Substrate solution (URT/ HR-US)	Amylase	according to US-A-URT	according to US-A-URT	according to US-A-URT	according to US-A-URT	according to US-A-URT	according to US-A-HR-US/ HR-US FTS
	Lipase	-	-	according to US-L-URT	according to US-L-HR-US	according to US-L-URT	according to US-L-HR-US
	Protease (free)	according to US-Pf-URT	according to US-Pf-URT	according to US-Pf-URT	according to US-Pf-HR-US	according to US-Pf-URT	according to US-Pf-HR-US/ HR-US FTS
	Protease (total)	-	-	according to US-Pt-URT	according to US-Pt-URT	-	according to US-Pt-URT/ HR-US FTS
	Trypsin (free)	according to US-Tf-URT	according to US-Tf-URT	according to US-Tf-URT	-	-	-
Enzyme solution (URT/ HR-US)	Amylase	according to US-A-URT (frozen)	according to US-A-URT (fresh/ frozen)	prepared in phosphate buffer 0.2 M (URT/ HR-US), URT (fresh/ frozen)	according to US-A-URT	according to US-A-URT	according to US-A-HR-US/ HR-US FTS (2.5 % EK)
	Lipase	-	-	according to US-L-URT (fresh/ frozen)	according to US-L-HR-US	according to US-L-URT	according to US-L-HR-US
	Protease (free)	according to US-Pf-URT (frozen)	according to US-Pf-URT (fresh/ frozen)	according to US-Pf-URT (fresh/ frozen)	according to US-Pf-HR-US	according to US-Pf-URT	according to US-Pf-HR-US/ HR-US FTS
	Protease (total)	-	-	according to US-Pf-URT (fresh/ frozen)	according to US-Pt-URT	-	according to US-Pt-URT/ HR-US FTS
	Trypsin (free)	according to US-Tf-URT (frozen)	according to US-Tf-URT (frozen)	Prepared in 1 mM HCl (without Triton X 100), URT (frozen)	-	-	-
Pharmacopoeia (no separation of fibers)	Amylase	-	-	according to 2.2.2.1 (Ph.Eur.) (only 3 h/ 7 h)	according to 2.2.2.1 (Ph.Eur.)	according to 2.2.2.1 (USP)	according to 2.2.2.1 (Ph.Eur.)
	Lipase	-	-	according to 2.2.2.2 (Ph.Eur.) (only 3 h/ 7 h)	according to 2.2.2.2 (Ph.Eur.)	according to 2.2.2.2 (USP)	according to 2.2.2.2 (Ph.Eur.)
	Protease (free)	-	-	according to 2.2.2.3 (Ph.Eur.) (only 3 h/ 7 h)	according to 2.2.2.3 (Ph.Eur.)	according to 2.2.2.3 (USP)	according to 2.2.2.3 (Ph.Eur.)
	Protease (total)	-	-	according to 2.2.2.4 (Ph.Eur.) (only 3 h/ 7 h)	according to 2.2.2.4 (Ph.Eur.)	-	according to 2.2.2.4 (Ph.Eur.)
	Trypsin (free)	according to 2.2.2.5 (Ph.Eur.)	-	according to 2.2.2.5 (Ph.Eur.)	-	-	-
Number of experiments (replicates) (Pharmacopoeia)		2 (2 rep)	-	2 (2 rep)	2 (2 rep)	6 (1 rep) on two days	2 (2 rep)

In order to evaluate repeatability and intermediate precision, the CVs for ultrasonic velocity changes for the six measurements were calculated (no calibration with working standard for determination of repeatability or intermediate precision).

Linearity tests were performed by using differently concentrated working standard solutions measuring two samples per concentration (two replicates each).

In order to evaluate **robustness** of methods, temperature, concentration and stability of analytical solutions, extraction time, activation parameters (total proteases) and parameters for emulsion preparation (lipases) were investigated. Robustness measurements were carried out by measuring two enzyme solutions (two replicates each) per tested condition.
In order to determine pancreatic enzyme activity in porcine pancreas and porcine pancreas powder-containing samples, defined sequences of enzyme solutions (working standard and samples) were measured, as shown in Table 12.

2.2.1.6 Application of titration equipment to HR-US 102 P

The following information regarding material of the titration equipment (Sonas Technologies Ltd., Dublin, Irleand) was provided:

- Tubing: PEEK (polyetheretherketone)
- Stirring tale: PEEK (polyetheretherketone)
- Valve: PTFE (polytetrafluorethylene)
- Syringe lock: Polypropylene

PEEK is known as biocompatible. Using the titration equipment, the dosing cap was connected via a tube (length 0.6 m) with a syringe (Microliter, Hamilton AG, Bonaduz, Switzerland), located in the dosing apparatus (Syringe dispenser PB-600^{-1} (Hamilton AG, Bonaduz, Switzerland) and filled with enzyme solution. The system was rinsed to remove air bubbles. The cell, filled with substrate, was closed with the dosing cap. Mixing was carried out by a small stirring tale, fixed at the dosing cup.
A linearity experiment was carried out to test the enzyme stability in the system. Periodic injections using the titration equipment and a syringe dispenser (Hamilton) were compared with experiments in which the enzyme solution was injected only once. In periodic intervals, 4 µl of the enzyme solution were added to the cell via titration equipment. In comparison, defined volumes of enzyme solution were injected into freshly prepared substrate solution once. When using periodic injection, the enzyme solution remained in the titration system for different time intervals before it was added to the cell. To control the enzyme stability, single injections were done with enzyme solution that had been kept in the dosing system for different time intervals.

2.2.1.7 Calibration with reference standard

In order to determine enzyme activity, ultrasonic measurements were performed with sample and reference standard. For one-point calibration, sample and reference standard weights were

chosen to obtain approx. identical slopes for both measurements. Generally, three independent reference standard solutions (two replicates each) were measured. Activities of unknown samples were calculated via rule of proportion using the average slopes of reference standard and sample measurements and the activity label claim of the reference standard. For three-point calibration, three independent reference standard solutions of different concentrations (within tested linear range) were measured (two replicates). A calibration line was calculated via linear regression. Using the linear equation of the calibration line and the activity label claim of the reference standard the activity in the sample was calculated.

2.2.1.8 Equations

Linear slope

The equation of a line is $y = mx + b$. The linear slope m was calculated as follows:

$$m = \frac{\sum(x - \bar{x}) \cdot (y - \bar{y})}{\sum(x - \bar{x})^2}$$

$\bar{x}, \bar{y}$ Means of x-, y-values

Arithmetic mean

The arithmetic mean $\bar{x}$ was calculated as follows.

$$\bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i$$

n Number of values

Standard deviation (stdev)

The standard deviation s was calculated as follows:

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{(n-1)}}$$

$\bar{x}$ Mean of x-values

n Number of values

Coefficient of Variation (CV)

The CV was calculated as follows:

$$CV = \frac{s}{\bar{x}} \cdot 100\,\%$$

$\frac{s}{}$ Standard deviation

$\bar{x}$ Mean of x-values

Percentage deviation D (of enzyme activity determined by using ultrasonic spectroscopy in relation to activity based on reference method (pharmacopoeia))

D was calculated as follows:

$$D = \frac{\left(\left|A_{US} - A_{RM}\right|\right)}{A_{RM}} \cdot 100\,\%$$

A_{US} Enzyme activity (determined by using ultrasonic spectroscopy) (u/g)

A_{RM} Enzyme activity (determined by using reference method (pharmacopoeia)) (u/g)

2.2.2 Determination of enzyme activity in pancreas powder according to pharmacopoeias

2.2.2.1 Determination of amylase activity in pancreas powder (Ph.Eur. and USP)

Four 250 ml conical flasks were marked (S, U, BS and BU). Starch solution 1 % (Ph.Eur./ USP) (25 ml), 10 ml of Phosphate buffer 0.2 M pH 6.8 (Ph.Eur./ USP) and 1 ml of Sodium chloride solution 0.2 M (Ph.Eur./ USP) were pipetted into each flask. After inserting the stoppers, the contents were mixed. The flasks were placed in a water bath maintained at 25 ± 0.1°C to equilibrate (Huber variostat CC, Peter Huber Kältemaschinenbau GmbH, Offenburg, Germany). Hydrochloric acid 1 M (2 ml) were added to BU and BS. After mixing the flasks were returned to the water bath. Amylase sample solution (1.0 ml) was added to U and BU, 1.0 ml of amylase reference solution was added to S and BS. After mixing, the flasks were returned to the water bath. After 10 min, accurately timed from the addition of the enzyme, 2 ml portions of 1 M hydrochloric acid were added to the flasks S and U followed by mixing. Iodine solution 0.05 M (10.0 ml) were added to each flask with continuous stirring. Immediately, 45 ml of 0.1 M sodium hydroxide solution were added to each flask. The flasks were placed in the dark at a temperature between 15 and 25°C for 15 min. After addition of 4 ml of sulphuric acid (2 N according to USP, 20 % (v/v) according to Ph.Eur.), the excess of iodine was titrated with 0.1 M sodium thiosulfate solution to the disappearance of the blue colour (Piston burette Titronic universal, Schott Instruments Analytics GmbH, Mainz, Germany).

The amylase activity was calculated as follows in units per milligram:

$$100 \cdot (C_S / W_U) \cdot (V_{BU}-V_U)/ (V_{BS}-V_S)$$

in which C_S is the amylase activity of the reference standard solution ($u \cdot ml^{-1}$), W_U is the amount of pancreas powder taken (mg), and V_U, V_S, V_{BU} and V_{BS} are volumes of 0.1 N sodium thiosulfate consumed in the titration of the solutions in flasks U, S, BU and BS, respectively.

2.2.2.2 Determination of lipase activity in pancreas powder (Ph.Eur. and USP)

Olive oil stock emulsion (Ph.Eur./ USP) (10 ml) and 19 ml of Reagents mixture (Ph.Eur./ USP) were filled in a jacketed glass vessel of about 50-ml capacity, the outer chamber of which was connected to a thermostatically controlled water bath (Huber ministat CC, Peter Huber Kältemaschinenbau GmbH, Offenburg, Germany). The mixture was covered and stirred continuously with a mechanical stirring device. With the mixture maintained a temperature of 37°C ± 0.1 °C, 0.1 M sodium hydroxide solution, from a auto microburet inserted through an opening in the cover, was added to adjust the pH to 9.0 (Ph. Eur.) or 9.2 (USP), respectively, potentiometrically using a calomel-glass electrode system. Lipase solution (Ph.Eur./ USP) was added (1.0 ml). Then, the addition of 0.1 M sodium hydroxide solution was continued for 5 min to maintain the pH at 9.0 (718 STAT Titrino, Metrohm AG, Herisau, Switzerland). The volume of 0.1 M sodium hydroxide solution after each min was determined.

The lipase activity was calculated as follows in units per milligram:

$$\frac{n \cdot m_1}{n_1 \cdot m} \cdot A$$

n	Average volume of 0.1 M sodium hydroxide solution used per min during titration of the test solution in ml
n_1	Average volume of 0.1 M sodium hxdroxide solution used per min during the titration of the reference solution in ml
m	Mass of substance to be examined in mg
m_1	Mass of reference solution in mg
A	Activity of pancreas powder reference in Ph.Eur.-units per mg

2.2.2.3 Determination of total protease activity in pancreas powder (Ph.Eur.)

Tubes in duplicate were marked T, Tb (samples), S_1, S_{1b}, S_2, S_{2b}, S_3, S_{3b} (standard series), one tube was marked B. Borate buffer solution pH 7.5 (Ph.Eur.) was added as follows:

B:	3.0 ml
S_1 and S_{1b}:	2.0 ml
S_2, S_{2b}, T and T_b:	1.0 ml

Reference solution (Total protease solution (Ph.Eur.)) was added to the tubes as follows:

S_1 and S_{1b}:	1.0 ml
S_2 and S_{2b}:	2.0 ml
S_3 and S_{3b}:	3.0 ml

Test suspension (Total protease solution (Ph.Eur.)) were added to tubes T and Tb (2.0 ml). 5.0 ml of Trichloroacetic acid solution (Ph.Eur.) were added to tubes B, S_{1b}, S_{2b}, S_{3b} and T_b and mixed by shaking. Tubes and the Casein solution (Ph.Eur.) were placed in a water bath at 35°C ± 0.5 °C, burdened by a glass rod or ball. When temperature equilibrium was reached, 2.0 ml of the Casein solution (Ph.Eur.) were added to the tubes B, S_{1b}, S_{2b}, S_{3b} and T_b. At time zero, casein solution was added successively and at intervals of 30 sec to tubes S_1, S_2, S_3 and T. Immediately after each addition the content was mixed. Exactly 30 min after addition of the Casein solution (Ph.Eur.) 5.0 ml of Trichloroacetic acid solution (Ph.Eur.) was added to tubes S_1, S_2, S_3 and T and the contents were mixed. The tubes were withdrawn from the water bath and allowed to stand at room temperature for 20 min.

The contents of each tube were filtered. Absorbance of the filtrates was measured at 275 nm using the filtrate obtained from tube B as compensation liquid (Lambda 25 UV/ Vis spectrometer, Perkin Elmer, Waltham, USA). The average of absorbance values for the filtrates obtained from tubes S_1, S_2, S_3 were corrected by subtracting the average values obtained for the filtrates from tubes S_{1b}, S_{2b}, S_{3b} respectively. A calibration curve was drawn of the corrected values against the volume of used reference solution.

Using the corrected absorbance for the test suspension (T, Tb) and the calibration curve and taking into account the dilution factors, the activity of the substance was determined. The test was not valid unless the corrected absorbance values were between 0.15 and 0.60.

2.2.2.4 Determination of free protease activity in pancreas powder (USP)

The procedure was similar to the determination of total protease activity (Ph.Eur.). Differences are described in the following.

Phosphate buffer solution pH 7.5 (USP) was added as follows:

B:	3.0 ml
S_1 and S_{1b}:	2.0 ml
S_2, S_{2b}, T and T_b:	1.5 ml
S_3 and S_{3b}:	1.0 ml

Reference solution (Free protease solution (USP)) was added to the tubes as follows:

S_1 and S_{1b}:	1.0 ml
S_2 and S_{2b}:	1.5 ml
S_3 and S_{3b}:	2.0 ml

Test suspension (Free protease solution (USP)) were added to tubes T and Tb (1.5 ml). Trichloroacetic acid solution addition was performed as described for the determination of total protease activity (Ph.Eur.).

Tubes and the casein solution were placed in a water-bath at 40°C, burdened by a glass rod or ball. When temperature equilibrium was reached, 2.0 ml of the Casein solution (USP) were added to each tube. Exactly 60 min after addition of the Casein solution (USP), 5.0 ml of trichloroacetic acid solution (USP) was added to tubes S_1, S_2, S_3 and T and the contents were mixed. The tubes were withdrawn from the water bath and allowed to stand at room temperature for 10 min.

The contents of each tube were filtered. Absorbance of the filtrates was measured at 280 nm using the filtrate obtained form tube B as compensation liquid (Lambda 25 UV/ Vis spectrometer, Perkin Elmer, Waltham, USA). Evaluation was done as described for the determination of total protease activity (USP).

2.2.2.5 Determination of trypsin activity in pancreas powder (Ph.Eur.)

Borate buffer 0.0015 M pH 8.0 (30.0 ml) and 3.0 ml of BAEE solution (Ph.Eur.) were filled in a jacketed glass vessel of about 50-ml capacity, the outer chamber of which was connected to a thermostatically controlled water bath (Huber ministat CC, Peter Huber Kältemaschinenbau GmbH, Offenburg, Germany). The mixture was covered, and stirred continuously with a mechanical stirring device. With the mixture maintained a temperature of 25°C, 0.02 M sodium hydroxide solution, from an auto microburet inserted through an opening in the cover, was added to adjust the pH to 8.0 potentiometrically using a calomel-glass electrode system. Trypsin solution (Ph.Eur.) was added (750 µl). Then, the addition of 0.02 M sodium hydroxide solution was continued for 5 min to maintain the pH at 8.0 (718 STAT Titrino, Metrohm AG, Herisau, Switzerland). The volume of 0.02 M sodium hydroxide solution after each min was determined.

The trypsin activity was calculated as follows in units per milligram:

$$\frac{n \cdot m_1}{n_1 \cdot m} \cdot A$$

n	Average volume on 0.02M sodium hydroxide solution used per min during titration of the test solution in ml
n_1	Average volume of 0.02 M sodium hxdroxide solution used per min during the titration of the reference solution in ml
m	Mass of substance to be examined in mg
m_1	Mass of reference solution in mg
A	Activity of trypsin reference in Ph.Eur.-units per mg

2.2.3 Emulsion preparation and characterization (Lipase)

2.2.3.1 High-pressure homogenization

In order to determine lipase activity by means of ultrasonic measurements, a stable substrate preparation was required. Stable emulsions can be processed in high-pressure homogenizers. They generally function as follows: A high pressure pump compresses the pre-emulsion to the desired homogenization pressure. Afterwards the emulsion expands in the homogenization valve. Thereby, the droplets of the pre-emulsion are reduced in the homogenization nozzle (Schubert, 2005). High pressure homogenizers are classified according to their kind of nozzle: Radial diffuser, aperture plate, jet disperser or opposed jet disperser (Schubert, 2005).
In this work, a Microfluidizer®110-EH processor (Microfluidics Intern. Corp., MA-Newton, USA), with an opposed jet disperser was used to prepare emulsions with high pressure.
The pre-emulsion was pressed through micro channels within an interaction chamber. A special fixed geometry leads to small droplet sizes, tight particle size distributions and, consequently, to a high stability of the emulsion. Changes in chamber type, channel diameter, process pressure and number of passages influence particle size and distribution.

Two main general types of chambers are available named according their geometry:

- In ‚z‘ chambers the channels feature a zigzag form (Figure 10 A) in which the product zigzags through the interaction chamber
- In ‚y‘ chambers the form resembles the alphabetic character (Figure 10 B) in which the product stream is divided into two streams that impinge upon each other (Küchler *et al.*, 2006).

Table 13. Overview of methods used for the preparation of olive oil emulsions with Microfluidizer® and performed analytics to evaluate the emulsion quality.

M	Reagents	Performance	Analytics
Ia	Olive oil stock emulsion (Ph.Eur./ USP): 10 ml Reagents mixture (Ph.Eur./ USP): 19 ml	Olive oil stock emulsion (Ph.Eur./ USP) was used as pre-emulsion. The pre-emulsion was homogenized with high-pressure (690 – 1200 bar) for four passages using a Microfluidizer equipped with F12Y/ F20Y (upstream) and H30Z (downstream). Reagents mixture (Ph.Eur./ USP) was added after high-pressure homogenization	Laser diffraction (Horiba LA 950); Baseline check using URT
Ib		Preparation of the Olive oil stock emulsion (Ph.Eur./ USP) was performed by using a Power propeller mixer (Eurostar with four-blade propeller stirrer 50/ 2000 (IKA Werke GmbH&Co.KG, Staufen, Germany) using 800 to 950 rpm for 15 min (pre-emulsion). Afterwards, the pre-emulsion was homogenized with high-pressure (900 - 1200 bar) for four passages using a Microfluidizer equipped with F12Y/ F20Y chamber (upstream) and H30Z chamber (downstream). Reagents mixture (Ph.Eur./ USP) was added after high-pressure homogenization	Laser diffraction (Horiba LA 950); Baseline check using URT
IIa	Olive oil stock emulsion (Ph.Eur./ USP): 100 ml Reagents mixture (Ph.Eur./ USP): 190 ml	Ingredients of the Olive oil stock emulsion (Ph.Eur./ USP) and Reagents Mixture (Ph.Eur./ USP) were mixed by using a Power propeller mixer (Eurostar with four-blade propeller stirrer 50/ 2000 (IKA Werke GmbH&Co.KG, Staufen, Germany) with 950 rpm for 15 min (pre-emulsion). Afterwards, the pre-emulsion was homogenized with high-pressure (1200 bar) for four passages using a Microfluidizer equipped with F20Y chamber (upstream) and H30Z chamber (downstream).	Laser diffraction (Horiba LA 950); Baseline check using URT
IIb		Olive oil stock emulsion (Ph.Eur./ USP) was diluted with Reagents mixture (Ph.Eur./ USP) (pre-emulsion). Afterwards, the pre-emulsion was homogenized with high-pressure (1200 bar) for up to 12 passages using a Microfluidizer equipped with F20Y chamber (upstream) and H30Z chamber (downstream).	Laser diffraction (Horiba LA 950); Baseline check using URT; Lipase activity measurements (URT)
III	Olive oil stock emulsion (Ph.Eur./ USP): 350 ml Reagents mixture (Ph.Eur./ USP): 665 ml	Stock emulsions were mixed for 5 or 15 min. Olive oil stock emulsion (Ph.Eur./ USP), containing 10 % olive oil and stock emulsions containing 6.3 % and 2.5 % olive oil (deviant volumes were compensated with water) were diluted with Reagents mixture (Ph.Eur./ USP) (pre-emulsion). Afterwards, the pre-emulsions were homogenized with high-pressure (600 - 1800 bar) for 1 to 5 passages using a Microfluidizer equipped with F20Y chamber (upstream) and H30Z chamber (downstream). The experiments were performed on two days.	Laser diffraction (Horiba LA 950); Baseline check using URT; Lipase activity measurements (URT)
IV	TRIS buffer (URT/ HR-US) for determination of Trypsin activity, Triton X 100, olive oil, Reagents mixture (Ph.Eur./ USP)	Triton X 100 (5mM) was dissolved in TRIS buffer (URT/ HR-US) for determination of Trypsin activity. After addition of olive oil (6 % (w/v)), the pre-emulsion was prepared by mixing in a household blender (BL 450, Kenwood Limited, Havant, United Kingdom) for 15 min (grade 1). Afterwards, the pre-emulsion was homogenized with high-pressure (1200 bar) for 3 passages using a Microfluidizer equipped with F12Y chamber (upstream) and H30Z chamber (downstream). Reagents mixture (Ph.Eur./ USP) was added after high-pressure homogenization in equal parts.	Laser diffraction (Horiba LA 950); Baseline check using URT; Lipase activity measurements (URT)
V	TRIS buffer (1-4) containing 5 mM Triton X 100, 3 % (w/v) olive oil, Reagents mixture (Ph.Eur./ USP) (1)/ Bile salts in TRIS buffer (2-4)	(1) Pre-emulsion was prepared in 100 mM TRIS buffer pH 7.8 (37 °C) containing 240 mM $CaCl_2$. After high-pressure homogenization (1200 bar, 3 passages) dilution with Reagents mixture (Ph.Eur./ USP) in equal parts, equivalent to final concentrations of approx. 50 mM TRIS and 120 mM calcium. (2) Pre-emulsion was prepared in 100 mM TRIS buffer pH 7.8 (37 °C) containing 240 mM $CaCl_2$. After high-pressure homogenization (1200 bar, 3 passages) dilution with bile salts solution (0.84 % (w/v) in TRIS 100 mM pH 7.8 with 240 mM $CaCl_2$) in equal parts, equivalent to final concentrations of approx. 100 mM TRIS and 240 mM calcium. (3) Preparation according to (2), but the used buffer system contained only 120 mM $CaCl_2$, equivalent to approx. 100 mM TRIS and 120 mM calcium. (4) Preparation according to (3), but the buffer solution contained 240 mM $CaCl_2$, equivalent to final concentrations of approx. 100 mM TRIS and 240 mM calcium.	Baseline check using URT; Lipase activity measurements (URT); pH measurement during lipase reaction (Equipment as used for determination of lipase activity according to Ph.Eur./ USP)

Due to the small diameter and the geometry, the product stream accelerates to high velocities. High shear forces caused by the fixed geometry and impact forces resulting from collisions of

the particles with walls and themselves are important for the intense particle size reduction. High turbulences play a major role (Küchler *et al.*, 2006). The disruption is primarily caused by inertial forces in turbulent stream (Schubert, 2005).
Additionally to the interaction chamber (IXC), an auxiliary processing module (APM), which is always a 'z' chamber, can be placed in-line downstream. Back pressure generated in this way enhances the life time of the chamber and supports the particle disruption. Insertion of the APM upstream supports the pre-dispersion (Microfluidics, 2008). Nearly constant pressure and the fixed geometry of the micro channels lead to uniform results.

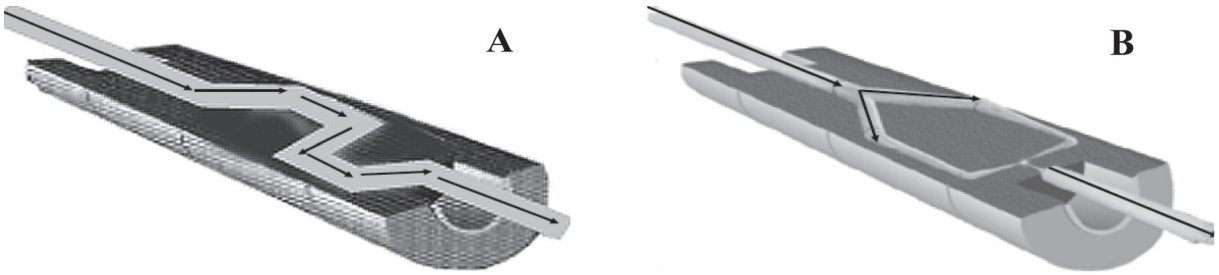

Figure 10. A Chamber type ‚z', B Chamber type 'y' (Microfluidics, 2008)

For the experiments, an F12Y or F20Y (75 µm minimum dimension) IXC was used, whereas an H30Z (200 µm minimum dimension) chamber was placed downstream as APM. During emulsion development operating pressures were varied from 500 to 1500 bar and number of passages from 1 to 12. The emulsions were cooled after each passage to maintain a temperature below 20°C. In Table 13 the different methods used during emulsion development are shown.

2.2.3.2 Determination of droplet size using static light scattering

To characterize and control the prepared emulsions, the Laser Scattering Particle Size Distribution Analyzer Horiba LA-950 (Retsch Technology GmbH, Haan, Germany) was used. It permits the determination of particle sizes in the range of 0.01 µm to 3000 µm.
An alternative common name for static laser scattering is laser diffraction. The instrument detects the correlation between the intensity and the angle of the light scattered from the particle.
At large particles (diameter > wavelength) the laser light is diffracted in small angles. The diffracted signals are captured in front relative to the light source. This phenomenon is described by the Fraunhofer theory. Optic properties of the investigated particles are irrelevant.
At small particles (several µm or less, diameter < wavelength) the laser light is scattered ascendingly in large angles. Therefore the scattered signals are captured at the sides and rear relative to the light source. This phenomenon is described in the Mie theory. For calculation of the particle size the diffraction and the absorption of the sample material was essential.
The Horiba LA-950 consists of two light sources, a red laser diode (650 nm) and a blue light emitting diode LED (405 nm) and an array of photodiodes to detect the scattered light over a

wide range of angles. Using the automatic sampling system, few droplets of the emulsion were dispersed in 160 ml of water (stirrer: grade 4). Agglomerates were broken up by ultrasound during sample addition. The particle size distribution of the sample was automatically calculated using the Mie theory. The refraction index of olive oil of 1.469 (Song *et al.*, 2002) for the dispersed phase was taken into account. Salts in the continuous phase were neglected due to strong dilution. Consequently, properties of water for the continuous phase was adopted. For gum-acacia stabilized emulsions, the refraction index was set to 1.600. Iterations were 15 for acacia gum-stabilized emulsions and 150 for Triton X 100-containing emulsions. Each sample was determined in triplicate, and the mean was calculated.

2.2.3.3 Microscopic analysis of emulsions

As the pharmacopoeias stipulate microscopic analysis of the olive oil emulsions used for lipase assay, microscopy was also used for emulsion evaluation in this work. Microscopic analysis was done using a Microscope Cx41 (Olympus GmbH, Hamburg, Germany) in combination with a digital camera C-4040Z (Olympus GmbH, Hamburg, Germany). One ocular (zoom 10 x) and three objectives (zoom 10 x, 30 x, 100 x) were available. According to pharmacopoeia, 10 ml of the stock emulsion containing 10 % (v/v) olive oil were diluted to 100 ml with gum acacia solution. Emulsions containing no gum acacia were inspected without dilution. The diluted or undiluted emulsions were pipetted to the object plate (5 µl) and covered with a cover slip. Due to small droplet sizes the highest zoom (1000 x) was chosen to inspect the prepared emulsions.

2.2.4 Determination of solid content in proteolysis material

The solid content was determined in order to identify optimal precipitation conditions for processing after proteolysis. At a solid content of less than 15 %, the proteolysis was generally stopped. The proteolysis suspension (10 g) was weighed into a beaker (50 ml) and 5.5 ml of 85 % (w/w) isopropanol were added. After stirring for 1 min with a glass rod, fibers were removed. After addition of further 20 ml of 85 % (w/w) isopropanol, the suspension was stirred for 1 min using a magnetic stirrer. Two graduated centrifugation glasses were filled with 10 ml of the suspension and centrifuged for 2 min at 700 rpm at room temperature (Megafuge 1.0S, Heraeus Instruments GmbH, Hanau, Germany). The solid content was read off from the graduation.

3. RESULTS

3.1 Emulsion development for determination of lipase activities by ultrasonic spectroscopy

3.1.1 Emulsions containing olive oil and gum acacia based on pharmacopoeia

As described in an earlier section (1.4.5), ultrasonic measurements are very sensitive to emulsion instabilities. Therefore, an adequate stability of the emulsion was required. The aim of the studies described in this section was to prepare an emulsion which shows no effect on ultrasonic velocity during ultrasonic spectroscopic measurements. To judge whether an emulsion was suitable, a baseline check was done measuring the difference in velocity between the measurement cells, both filled with emulsion. Obtaining a stable difference between both cells indicated an emulsion of suitable stability. Pre-tests showed that the stability of the olive oil emulsion prepared in a blender according to pharmacopoeia was not sufficient to obtain stable baselines during ultrasonic velocity measurements. Thus, a high pressure homogenizer Microfluidizer® M-110 EH processor was used. In the majority of cases, the addition of reagents mixture after high-pressure homogenization of the olive oil stock emulsion led to instable baselines (Table 13, emulsification methods Ia and Ib). The droplet sizes enlarged with increasing number of passages immediately after first passage using emulsification method Ia (Table 13, household blender). Some of the emulsions prepared by using a propeller mixer (Table 13, emulsification method Ib) showed stable baselines. But using low rotations/ min, phase separation was observed immediately after stopping mixing or after filling the inlet reservoir of the homogenizer. Consequently, the amount of olive oil in the final emulsion was not defined. With increasing pressure and passages the droplet sizes increased (**Appendix A I**). Figure 11 shows the dependency of the droplet size on the quality of the pre-emulsion. The strong dependency on the rotations/ min proves low robustness of the method using the power propeller mixer for preparing the pre-emulsion (Table 13, emulsification method Ib). Working with low pressures, the enlargement of droplet size with ascending numbers of passages was reduced (Figure 12). Changing the interaction chamber F12Y against a JR20Z, the enlargement of droplet size was also minimized (**Appendix A I**).

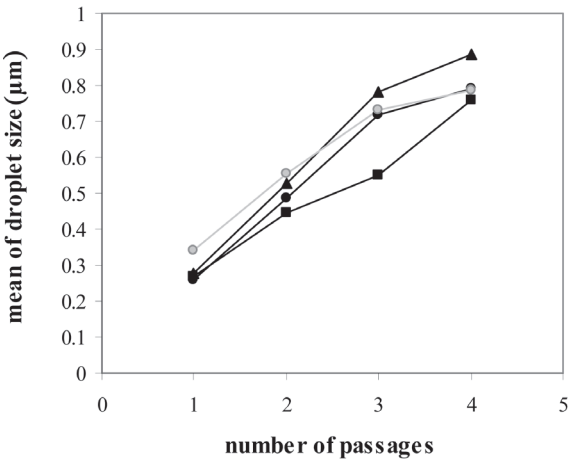

Figure 11. Influence of pre-emulsification on droplet size of an emulsion of olive oil in water. Pre-emulsions were prepared using a propeller mixer at 800 rpm (square), 850 rpm (triangle) or 950 rpm (circle) for 15 minutes. The Microfluidizer was operated at 1200 bar with a F12Y-chamber upstream and a H30Z-chamber downstream (four passages each). The grey-marked series represents data obtained with double volume of emulsion during pre-emulsification (950 rpm). Shown are means of 3 replicates from a single experiment. Standard deviations are below 0.01 µm.

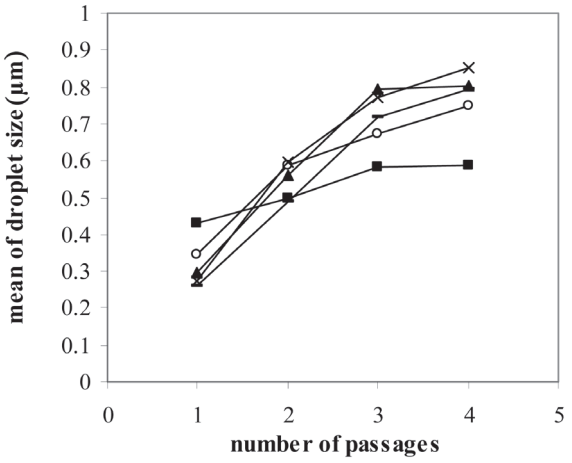

Figure 12. Influence of homogenization pressure on droplet size of an emulsion of olive oil in water. Pre-emulsions were prepared using a propeller mixer at 950 rpm.The Microfluidizer was operated at 900 bar (square), 1000 bar (triangle), 1100 bar (circle), 1200 bar (line), or 1500 bar (cross) with a F12Y-chamber upstream and a H30Z-chamber downstream (four passages each). Shown are means of 3 replicates from a single experiment. Standard deviations are below 0.01 µm.

Addition of bile salts solution before high pressure homogenization was done by using emulsification methods IIa and IIb (Table 13). The pre-emulsion prepared with emulsification method IIa was very instable. Phase separation was observed immediately after the propeller mixer was stopped. After microfluidization all passages showed stable baselines in the ultrasonic spectrometer (URT). The signal after enzyme injection was higher than in earlier tests. The means of droplet diameter showed high values, but a decrease of droplet size was observed with increasing number of passages. The content of oil was not defined due to phase separation in the inlet reservoir of the Microfluidizer®. Using emulsification method IIb, again the emulsions from all passages showed stable baselines and the droplet diameter decreased with increasing number of passages. In a second trial more passages were performed using the same conditions. (**Appendix A II**).

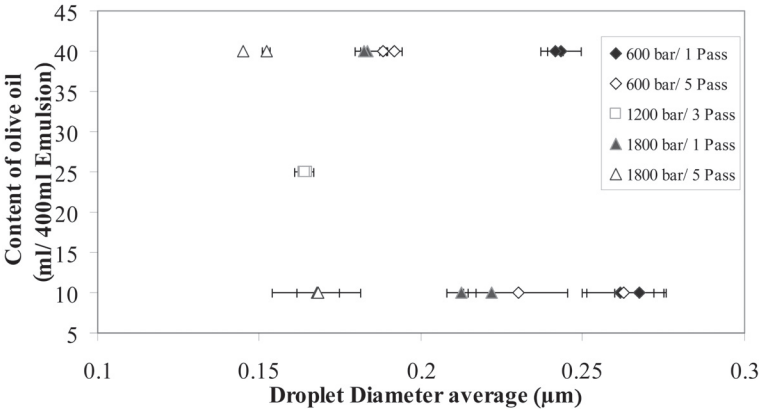

Figure 13. Influence of olive oil content, homogenization pressure and number of passages on droplet size of an emulsion of olive oil in water. Olive oil stock emulsions were diluted with Reagents mixture (pre emulsion). The Microfluidizer was operated at 600 to 1800 bar with a F20Y-chamber upstream and a H30Z-chamber downstream (1 to 5 passages). Shown are means of 3 replicates from two experiments. Pre-emulsification times of 5 and 15 min are not distinguishable from each other due to equal symbol forms and colors.

Additional experiments were done considering content of olive oil, homogenization pressure, number of passages and time of emulsification of stock emulsions (emulsification method III). Complete data are shown in **Appendix A II**. In Figure 13, the results are summarized. More passages caused smaller droplet diameters for high as well as low content of olive oil. High pressure led also to smaller droplet diameters for high as well as low content of olive oil. High standard deviations were observed for emulsions with a low content of olive oil (2.5 % olive oil in stock emulsion). Intermediate preparation conditions (10 min emulsification of stock emulsion, olive oil content 6.3 %, homogenization pressure 1200 bar) led to highest reproducibility with respect droplet sizes. The time of mixing the stock emulsions showed no significant effect on the droplet size. The ultrasonic signal after addition of working standard solution decreased with increasing olive oil content. Although the emulsions with high olive oil content prepared with highest pressure showed an adequate reproducibility in droplet size, they were not considered to be best due to the little ultrasonic signal during lipase reaction and the high stress of the homogenizer. Concerning the high reproducibility regarding droplet size, the emulsions prepared with intermediate conditions (10 min emulsification of stock emulsion, olive oil content 6.3 %, homogenization pressure 1200 bar) were chosen for further experiments.
Calcium chloride was added to the emulsion either before or after microfluidization. The droplet size increased extremely with ascending calcium content. During ultrasonic measurements no stable baselines were observed using a calcium containing emulsion (**Appendix AII**). Furthermore, linearity was tested. Initially, the lipase emulsion was tempered at 37°C continuously during measurements. No linearity and high standard deviations were observed. Therefore, the experiment was repeated, but the emulsion was tempered only for 5 min separately for each measurement. Linearity was observed in the range of 0.02 to 0.18 % reference standard solutions (**Appendix AII**). Both tests showed that the stability towards calcium and temperature was limited.

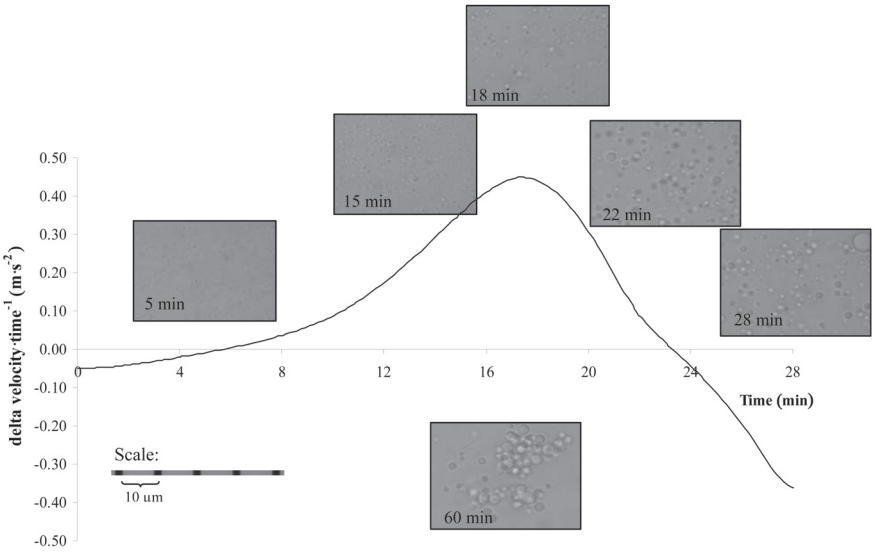

Figure 14. Ultrasonic and simultaneous microscopic observation of lipase reaction over time in an olive oil emulsion stabilized with gum acacia. The emulsion was prepared with a Microfluidizer. Both measurement cells (URT) were filled with emulsion (180µl) After checking stability of the baseline, 4 µl of working standard solution (1.0 % (w/v)) were added to one of the cells. The measurement was carried out at 30°C.

Figure 14 shows the time dependency of ultrasonic velocity after enzyme injection. Simultaneously, a second sample was monitored outside the instrument by microscopy. The change of ultrasonic velocity after approx. 18 min did not follow a general enzyme reaction curve. Rather, an extreme decrease of ultrasonic velocity occured after 18 min, accompanied by a fast droplet growth with a final emulsion breaking.

In order to ascertain the influence of the sample matrix (proteases) on the gum acacia-stabilized emulsion, 5 % (w/v) gum acacia was added to 0.3 % (w/v) casein solution. Working standard solution (1.0 % (w/v)) was injected into casein solution in the presence or absence of 5 % gum acacia (Figure 15). In the gum acacia-containing casein solution the protease activity was minimally enhanced. The degradation of gum acacia by pancreatic proteases can be suggested.

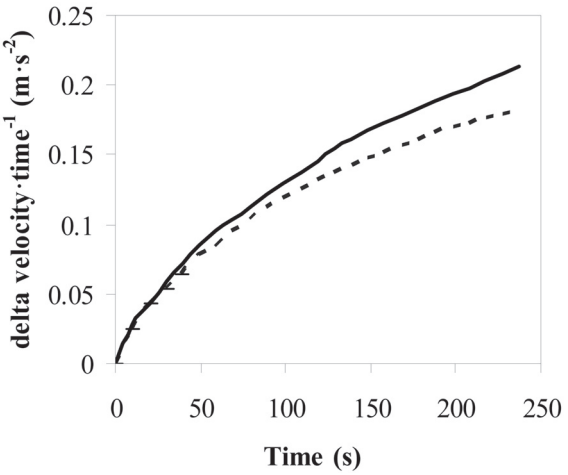

Figure 15. Influence of gum acacia on the protease reaction in 0.3 % (w/v) casein. URT-measurements were done in the presence (dashed) and the absence (continuous) of gum acacia (5 % (w/v)) at 37°C. Working standard solution (1.0 %, 4 µl) was injected into 180 µl substrate solution

3.1.2 Emulsions containing olive oil and Triton X 100

Using Triton X 100 as emulsifier, the pre-emulsion was prepared in TRIS buffer (URT/ HR-US) for determination of Trypsin activity. After high-pressure homogenization, the emulsion was diluted with Reagents mixture (Ph.Eur./ USP) (Table 13, emulsification method IV). The ultrasonic velocity during lipase reaction showed a regular course over time in opposite to the gum acacia-stabilized emulsions. In order to test quality of the emulsion and suitability for the ultrasonic spectroscopic determination of lipase activity, several parameters were varied. Lipase activities as well as droplet sizes were determined for the emulsions. In the following section, the modal means of droplet size are presented instead of arrithmetic means because diminutive second maxima at higher particle diameters were observed, supposably caused by impurities in the measuring cell of the Horiba LA 950.

Repeatability

The repeatability was 3.08 %.

Number of passages

The number of passages was varied from one to four passages. From the first to the second passage a reduction of modal droplet size was observed. More passages led to no further decrease. Initial slopes were unaffaceted by the number of passages (**Appendix A III**).

Concentration of olive oil

The olive oil concentration in the homogenized emulsion was varied from 1 % to 6 % (v/v), equivalent to final concentrations of 0.5 % to 3 % (v/v) after dilution with bile salts solution (Figure 16). No further increase of the initial slopes was observed at concentrations about 2 % (v/v). The droplet size increased with enhanced content of olive oil. A concentration of 6 % (v/v) olive oil, equivalent to 3 % (v/v) olive oil after dilution with bile salts solution was defined for the following experiments. Thus, the olive oil content used was equal to the final content in the pharmacopoeia emulsion (**Appendix A III**).

Concentration of Triton X 100

The concentration of Triton X 100 in the homogenized emulsion was varied from 1 to 7 mM, equivalent to a final concentration of 0.5 to 3.5 mM after dilution with bile salts solution. At concentrations higher than 3 mM, lipase activity reached a plateau.
A concentration of 5 mM was defined for further experiments. Droplet sizes decreased with increasing Triton X 100 concentration (**Appendix A III**).

Concentration of calcium chloride in emulsion

The calcium concentration in the emulsion was varied from 5 to 400 mM, equivalent to final concentrations of 2.5 to 200 mM (Figure 17). Saturation was achieved at concentrations above 100 mM (50 mM in final emulsion). No further increase of initial slopes was observed. The droplet size was not dependent on the content of calcium (**Appendix A III**).

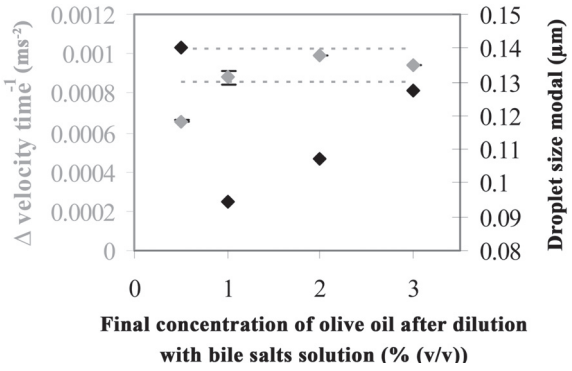

Figure 16. Influence of olive oil content in emulsion on ultrasonic velocity change (URT) and droplet size during lipase determination. Pre-emulsion was prepared in 100 mM TRIS pH 7.8 with 5 mM Triton X 100 and 20 mM calcium chloride. For homogenisation the Microfluidizer was operated at 1200 bar for 3 passages. Pre-emulsion and reagents mixture were mixed in equal parts (final emulsion). URT-measurements were carried out with 180 µl of final emulsion and 4 µl of working standard solution (0.3 %) at 37 °C. Left axis (grey) shows the inital slopes (URT): mean +/- stdev, n =2 (2 rep) (0.5 - 1 % olive oil), n =1 (2 rep) (2 - 3 % olive oil). Dashed lines are limits of +/- 3 s (s = 3.08%). Right axis (black) shows the modal droplet size (n = 1 (3 rep)).

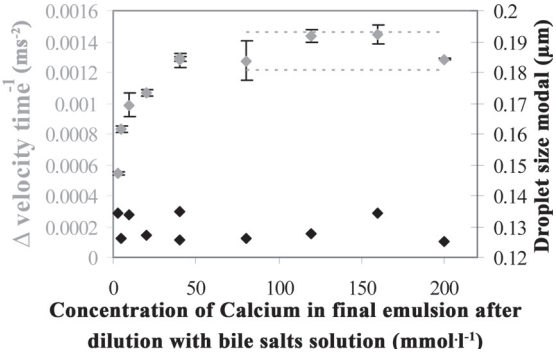

Figure 17. Influence of calcium content in emulsion on ultrasonic velocity change (URT) and droplet size during lipase determination. Pre-emulsion was prepared with 6 % (w/v) olive oil in 100 mM TRIS pH 7.8 with 5 mM Triton X 100. For homogenisation the Microfluidizer was operated at 1200 bar for 3 passages. Pre-emulsion and reagents mixture were mixed in equal parts (final emulsion). URT-measurements were carried out with 180 µl of final emulsion and 4 µl of working standard solution (0.3 %) at 37 °C. Left axis (grey) shows the inital slopes (URT): mean +/- stdev, n =2 (2 rep). Dashed lines are limits of +/- 3 s (s = 3.08%). Right axis (black) shows the modal droplet size (n = 1 (3 rep)).

Concentration of TRIS in emulsion

TRIS concentration was varied from 20 to 160 mM in the pre-emulsion before addition of bile salts solution. Further TRIS added via bile salts solution was not considered. At concentrations above 80 mM TRIS in the pre-emulsion, the initial slopes reached a plateau. An influence on the droplet size was not observed (**Appendix A III**).

Stability of emulsion

The pre-emulsion was stored over 10 days at 5 ± 3°C both after and before addition of the bile salts solution. In the latter case the bile salts solution was added freshly on the day of measuring. In the emulsion stored with bile salts, the activity was only minimally reduced after ten days (**Appendix A III**).

In order to optimize the buffering system, several emulsions were prepared with different buffers containing different concentrations of TRIS and calcium chloride (emulsification method V). Each emulsion was used for ultrasonic determination of lipase activity (URT). A second mixture was used to control the pH during enzyme reaction (Equipment as used for determination of lipase activity according to Ph.Eur./ USP). Results of ultrasonic and pH measurements are presented in **Appendix A IV**. Applying a total TRIS concentration of 50 mM, the change of ultrasonic velocity over time during lipase reaction was comparable to the course of the curve in a gum acacia-stabilized emulsion (Figure 14). Higher TRIS concentrations of 100 mM or 200 mM did not show such behavior. In the emulsion containing 50 mM TRIS, a decrease of pH of approx. 0.2 units within 15 min during lipase reaction was observed, whereas in the emulsions with higher TRIS content the pH decreased only minimally over time.

3.2 Feasibility study to determine pancreatic enzyme activity in drug substance by using ultrasonic velocity measurements

3.2.1 Specificity

As specificity is one important criterion for the evaluation of an analytical procedure, the specificity of enzyme activity measurements by ultrasonic methods was investigated for amylase, lipase, protease and trypsin (2.2.1.2 - 2.2.1.3, 2.2.1.5). In order to determine specificity, ultrasonic velocity change was measured in systems to which either substrate or enzym had not been added as well as in complete systems (positive control). For all enzymes ultrasonic velocity only changed in complete systems, but not in those that lacked substrate or enzyme. Thus, the methods were specific for enzyme activity detection. As an example, results of lipase determination are shown in Figure 18. Specificity tests for the other enzymes are accommodated in **Appendix A V**.

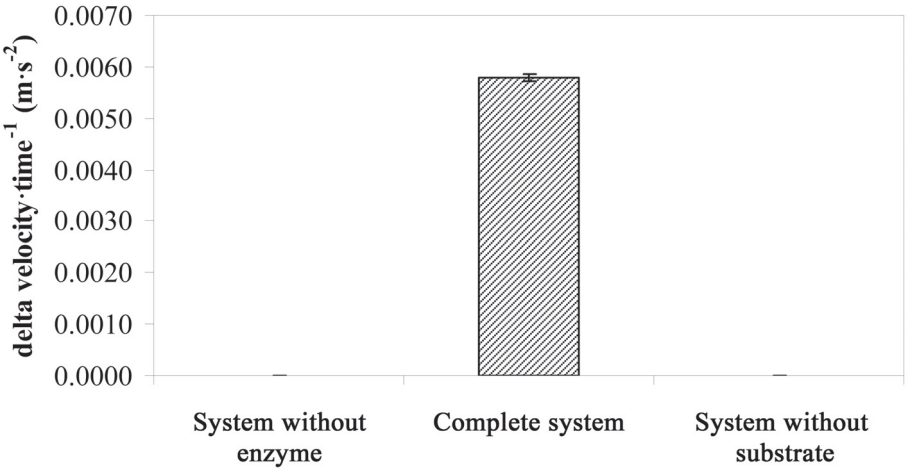

Figure 18. Specificity test for lipase activity measurement by ultrasonic method (HR-US). Measuring ultrasonic velocity change in the complete system (addition of 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of final olive oil emulsion (URT/ HR-US)) a clear change of ultrasonic velocity was observed. On the left, only 20 µl of Maleate buffer solution pH 7.0 (HR-US/ URT) were injected, on the right, a bile salts/ TRIS/ Triton X 100 mixture was used as "substrate". In both cases ultrasonic velocity did not change significantly. Shown are means +/- stdev (n = 2 (2 replicates)) to improve clarity of the figure.

Additionally, specificity was tested after adding substances to the working standard solutions, which were expected to be present in in-process samples (worst case).

The influence of sodium chloride was tested by adding sodium chloride to the sample solvents used for the determination of lipase and amylase activity. Sodium chloride added to the sample solvent showed no influence on lipase activity (Table 14, **Appendix A VI**), although sodium chloride is absolutely necessary for pancreatic lipolysis (Ruyssen & Lauwers, 1978). Thus, maleate buffer pH 7.0, which contains 40 mM sodium chloride, is used according to Ph.Eur method. In order to ensure a low salts concentration (Born *et al.*, 2009), the same buffer was used for the ultrasonic method, although the concentration of lipase solution (URT/ HR-US) was considerably higher as that according to Ph.Eur. According to Benzonana & Desnuelle (1968), sodium chloride supports the ionization of the fatty acids at pH 9. However, at a pH of 7.2 used for ultrasonic measurements ionization of fatty acids is less important. Higher concentrations of sodium lead to inhibition of lipase activity (Borgström & Erlanson, 1973, Ruyssen & Lauwers, 1978). Both activation and inhibition were not observed within the tests performed in the present thesis, so the method is evaluated as robust concerning sodium in the sample matrix. Concerning amylase, no activating effect of sodium has been reported in the literature, but Wakim *et al.* (1969) found an intense enhancement of amylase stability and activity caused by chloride ions. Starch solution (URT/ HR-US) contains 6.7 mM sodium chloride (Bernfeld, 1955). Although chloride is not directly added to the proteolysis make-up, it is likely to be introduced to the proteolysis make-up via glands. No influence of chloride in the sample matrix was observed (Table 14, **Appendix A VI**), so the method is evaluated as robust concerning chloride in the sample matrix.

Table 14. Influence of NaCl concentration in sample matrix on ultrasonic determination of amylase and lipase. Working standard solutions were prepared in the respective sample solvents with different NaCl content. Ultrasonic measurements were performed after adding the working standard solution to the respective substrate preparation at 37°C.

	Sample solvent	Sodium chloride concentration		Result
		According to developed method (2.1.3)	Tested range (sample solvent)	
Amylase	Phosphate buffer solution (URT/ HR-US)	6.7 mM	0 - 54 mM	No influence on amylase activity
Lipase	Maleate buffer solution pH 7.0 (URT/ HR-US)	40 mM	0 - 342 mM	No influence on lipase activity

Calcium is added to the proteolysis make-up in very low amounts. However, since calcium has an important function in the stabilization and activation of pancreatic enzymes, the effect of calcium in the sample matrix had to be tested. Adding calcium chloride to the solvents in higher amounts than expected in in-process samples showed no influence on the change of ultrasonic velocity during amylase, lipase, protease or trypsin reaction (Table 15, **Appendix A VI**).

In agreement with the ultrasonic method, the pharmacopoeias do not specify the addition of calcium to the amylase assay. In the literature, calcium is only considered to have a protective effect towards proteolytic attacks (Stein & Fischer, 1958) and a stabilizing effect (Vallee *et al.*, 1959; Buisson *et al.*,1987), but no influence on amylase activity. During emulsion development it was shown that lipase activity (change of ultrasonic velocity over time) did not increase further at calcium concentrations above 50 mM calcium in the olive oil emulsion (Figure 17). Calcium (60 mM) was used in the ultrasonic lipase method in order to avoid lag times between enzyme addition and maximum lipolysis rate, as described by Brown *et al.* (1977). Thus, analysis times could be reduced. Several authors (Schønheyder & Volqvartz, 1945; Scow, 1988) suggested a removal of free fatty acids by accumulation of calcium soaps to be responsible for the activation of lipase.

Table 15. Influence of $CaCl_2$ concentration in sample matrix on ultrasonic determination of pancreatic enzyme activities (amylase, lipase, total protease, trypsin). Working standard solutions in the respective sample solvents with different $CaCl_2$ content were prepared. Ultrasonic measurements were performed after adding the working standard solution to the respective substrate preparation at 37°C.

	Sample solvent	Calcium chloride concentration		Result
		According to developed method (2.1.3)	Tested range (sample solvent)	
Amylase	Phosphate buffer solution (URT/ HR-US)	-	0 - 3.35 mM	No influence on amylase activity
Lipase	Maleate buffer solution pH 7.0 (URT/ HR-US)	-	0 - 1000 mM	No influence on lipase activity
Protease (total)	Calcium chloride solution 0.02 M (URT/ HR-US)	20 mM	0 - 20 mM	No influence on total protease activity
Trypsin	Triton X 100 solution (URT/ HR-US)	-	0 - 300 mM	No influence on free trypsin activity

Table 16. Influence of isopropanol concentration (30 % (v/v), 25 % (w/w)) in sample matrix on ultrasonic determination of pancreatic enzyme activities (amylase, lipase, protease, trypsin). Working standard solutions in the respective sample solvents with different isopropanol content were prepared. Ultrasonic measurements were performed after adding the working standard solution to the respective substrate preparation at 37°C. (CV = Coefficent of variation).

	Sample solvent	Isopropanol concentration		Result
		According to developed method (2.1.3)	Tested range (sample solvent)	
Amylase	Phosphate buffer solution (URT/ HR-US)	-	0 - 30 % (v/v)	No influence on amylase activity
Lipase	Maleate buffer solution pH 7.0 (URT/ HR-US)	-	0 - 30 % (v/v)	No influence on lipase activity
Protease (free)	Borate buffer solution pH 7.5 (URT/ HR-US)	-	0 - 30 % (v/v)	No influence on free protease activity
Protease (total)	Calcium chloride solution 0.02 M (URT/ HR-US)	-	0 - 30 % (v/v)	No influence on total protease activity
Trypsin	Triton X 100 solution (URT/ HR-US)	-	0 - 30 % (v/v)	Small increase of initial slopes/ high CVs above 20 % (v/v) isopropanol

In the pharmacopoeia methods, the addition of calcium to the assay is not required. However, considering the calcium which is introduced passively via gum acacia, bile salts and pancreas powder a calcium concentration of approx. 5 mM in the pharmacopoeia assay can be assumed. As free fatty acids are titrated with sodium chloride, higher concentrations of calcium are not necessarily required in the pharmaocpoeiea assays. For ultrasonic measurements of proteases, calcium was added to the system via substrate (trypsin) or enzyme solution (total **protease**) in agreement with the pharmacopoeias. In the literature, calcium ions are described as essential for complete activation of trypsin and having furthermore stabilizing effects (Ruyssen & Lauwers, 1978). For the determination of free protease activity, calcium is not considered in the USP and was therefore omitted from the ultrasonic method. Taken together, the methods are evaluated as robust concerning calcium in the sample matrix.

Isopropanol content in the sample solvents up to 30 % (v/v) and 25 % (m/m), respectively, was tested. In-process samples of early process steps (API process) contain varying amounts of isopropanol. Pancreatic enzymes start to precipitate at isopropanol concentrations of approx. 30 % (w/w). During lipase, protease and amylase reaction, the change of ultrasonic velocity over time was not influenced by the presence of isopropanol concentrations up to 25 % (w/w) in the sample solvent (Table 16, **Appendix A VII**). Trypsin showed a slight increase of initial slopes accompanied by high CVs of up to 17 % at isopropanol concentrations above 16 % (w/w).

In-process material also harbors diverse proteins introduced via glands. In order to check the influence of the present proteins, casein, a mixture of several proteins, was utilized for simulation. Whereas porcine pancreas glands contain up to 20 % of proteins and solid components, for

Table 17. Influence of protein (casein) concentration in sample matrix on ultrasonic determination of amylase, lipase and trypsin. Working standard solutions in the respective sample solvents with different casein content were prepared. Ultrasonic measurements were performed after adding the working standard solution to the respective substrate preparation at 37°C.

	Sample solvent	Casein concentration		Result
		According to developed method (2.1.3)	Tested range (sample solvent)	
Amylase	Phosphate buffer solution (URT/ HR-US)	-	0 - 1.25 % (w/v)	Little increase of activity from 0 to 0.6 % (w/v) casein
Amylase	Phosphate buffer solution 0.2 M (URT/ HR-US)	-	0 - 1.25 % (w/v)	No influence on amylase activity
Lipase	Maleate buffer solution pH 7.0 (URT/ HR-US)	-	0 - 1.25 % (w/v)	No influence on lipase activity
Trypsin	Triton X 100 solution (URT/ HR-US)	-	0 - 1.25 % (w/v)	No influence on trypsin activity

early in-process samples, approx. one half can be assumed. In the course of proteolysis process, the content decreases steadily. Considering the presence of high-weighed fibers and sample dilution, the tested range from 0 to 2.5 % (w/v) casein should be sufficient. For the determination of amylase activity, a slight increase of ultrasonic velocity change over time was observed using Phosphate buffer (URT/ HR-US) when casein was present. This increase of ultrasonic signal could theoretically be caused by simultaneous monitoring of protease reaction as protein was available as substrate. However, the effect was eliminated by changing the phosphate buffer concentration in the enzyme solvent from 0.02 M to 0.2 M (Table 17, **Appendix A VIII**). Using starch solution also prepared in Phosphate buffer 0.2 M (URT/ HR-US), inadequate standard deviations were obtained during ultrasonic measurements of amylase activity, supposably caused by instability of the starch solution. Thus, the less concentrated Phosphate buffer (URT/ HR-US) was used to prepare the starch solution allowing reliable ultrasonic measurements. Lipase and trypsin activities were not influenced by the protein concentrations tested (Table 17, **Appendix A VIII**). For the tested range, a disturbance by proteins in the matrix (model casein) can be excluded for the ultrasonic determination of amylase (using Phosphate buffer 0.2 M (URT/ HR-US) as sample solvent), lipase and trypsin activity.

Process material contains a complex mixture from mono-, di- and triglycerides introduced via glands. Olive oil, a mixture of diverse glycerides, was used to simulate fat in the matrix. Whereas porcine pancreas glands contain approx. 15 % of lipids, for early in-process samples, approx. one half can be assumed. In the course of proteolysis process, the content decreases steadily. Olive oil concentrations in the range of 0 to 7.5 % were tested. The addition of olive oil in different concentrations had no or only a small influence on ultrasonic velocity change as a measure of lipase and protease activity (Table 18, **Appendix A IX)**.

Regarding the determination of amylase and trypsin activity, a strong influence of the addition of fat on the ultrasonic velocity changes was found. Using 0.02 M phosphate buffer

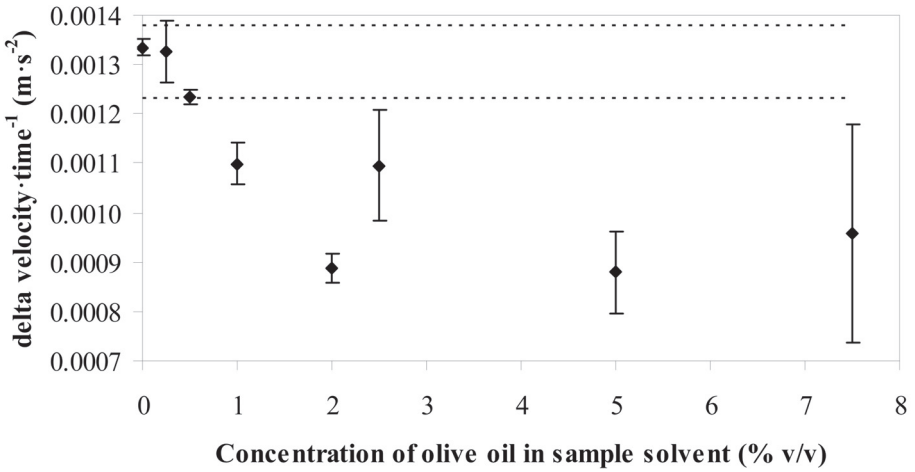

Figure 19. Influence of fat (olive oil) on amylase activity measurement by ultrasonic method (URT). Working standard solutions (0.05 %) with different olive oil content were prepared by mixing in a household blender for 10 min. Ultrasonic measurements were performed after adding 4 µl of working standard solution (0.05 %) to 180 µl of starch solution 6 % (URT/ HR-US) at 37°C. Shown are means +/- stdev (n = 2 (2 replicates)) to improve clarity of the figure. Dashed lines represent limits of +/- 3s (s = 1.86 %).

for the determination of amylase activity, a clear decrease of initial slopes was observed with ascending olive oil content. At olive oil concentrations higher than 2 % (v/v), high standard deviations occurred (Figure 19). Furthermore, blanks were determined by repeating the experiment, but only Phosphate buffer solution (URT/ HR-US) served as 'substrate'. The blanks showed an opposite behavior. In another experiment an increased phosphate buffer concentration (0.2 M) was introduced for the preparation of starch and amylase solution on the one hand and only for the preparation of amylase solution on the other hand. Ultrasonic measurements with starch solution prepared in the Phosphate buffer solution 0.2 M (URT/ HR-US) were unreliable due to high CVs. Preparing only the amylase solution in 0.2 M Phosphate buffer 0.2 M (URT/ HR-US) eliminated the influence of fat on the ultrasonic veloctiy change as a measure of amylase activtiy. But the dispersion was insufficient, as oil agglutinations were found at the mixer wall and the solvent emulsion showed only a slight turbidity (Table 12, **Appendix A IX**).

Due to the instability of the sample solvent emulsions, 0.2 % (v/v) of Triton X 100 was added in an additional experiment to the sample solvents for the determination of amylase (optimized) and total protease activity. The addition of Triton X 100 did not lead to a recurrence of fat disturbances regarding amylase and to an occurance of fat disturbances regarding total protease, respectively.

Using Triton X 100 solution (URT/ HR-US), for the determination of trypsin activity an extreme increase of initial slopes was observed (Figure 20, **Appendix A IX**). The results were confirmed by using official FIP reference standard solutions (grey triangles) instead of working standard solutions (Figure 20, **Appendix A IX**).

Blanks were determined by repeating the experiment, but only TRIS buffer (URT/ HR-US) for the determination of Trypsin activity served as 'substrate'. Blanks showed the same behavior towards fat, i.e. an increase of ultrasonic velocity change with ascending olive oil content. In a third experiment, Triton X 100 was omitted leading to an elimination of fat disturbances.

Additionally, other solvents were tested concerning applicability of oil-containing samples (Figure 21, Table 18). The use of borate buffer, phosphate buffer and calcium chloride solution showed no benefit compared to the use of Hydrochloric acid 1 mM (URT/ HR-US).

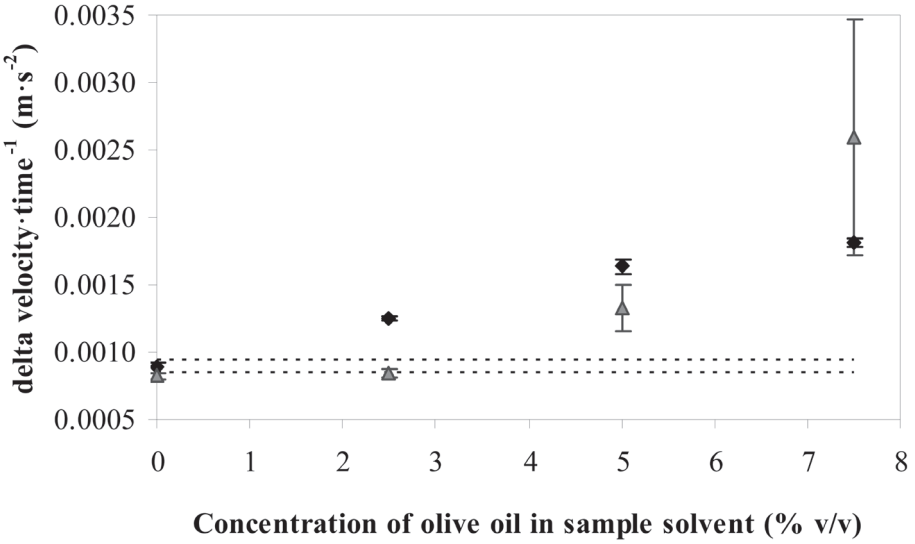

Figure 20. Influence of fat (olive oil) on trypsin activity measurement by ultrasonic method (URT). Working standard solutions (1.0 %, black squares) and official FIP trypsin reference standard solutions (0.03 %, grey triangles) with different olive oil content were prepared by mixing in a household blender for 10 min. Ultrasonic measurements were performed after adding 8 µl of the working/ FIP standard solution to 180 µl of BAEE solution (URT/ HR-US) at 37°C. Shown are means +/- stdev (n = 2 (2 replicates)) to improve clarity of the figure. Dashed lines represent limits of +/- 3s (s = 1.73 %).

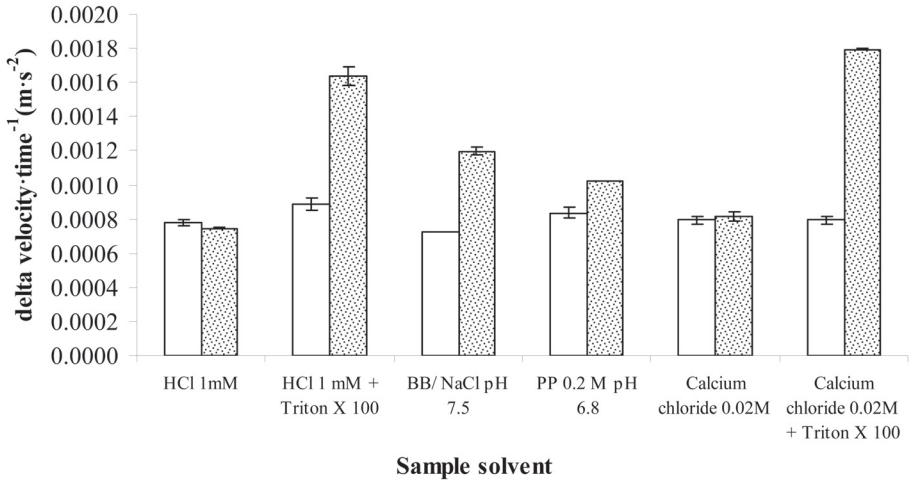

Figure 21. Test of several sample solvents (Borate buffer (BB), Phosphate buffer (PP) and $CaCl_2$ as alternative to HCl to avoid fat disturbances during ultrasonic determination of trypsin activity (URT). Working standard solutions (0.1 %) in different solvents with different olive oil content were prepared by mixing in a household blender for 10 min. Ultrasonic measurements were performed after adding 8 µl of working standard solution (0.1 %) to 180 µl of BAEE solution (URT/ HR-US) at 37°C. White columnes show the change of ultrasonic velocity without addition of olive oil, grey colmunes show the change of ultrasonic velocity in the presence of 0.5 % (v/v) olive oil in the sample solvent. Shown are means +/- stdev (n = 2 (2 replicates)) to improve clarity of the figure.

Table 18. Influence of fat concentration in sample matrix on ultrasonic determination of pancreatic enzymes. Working standard solutions in the respective sample solvents with different olive oil content were prepared by mixing in a household blender for 10 min. Ultrasonic measurements were performed after adding the working standard solution to the respective substrate preparation at 37°C.

	Sample solvent	Olive oil concentration		Result
		According to developed method (2.1.3)	Tested range (sample solvent)	
Lipase	Maleate buffer solution pH 7.0 (URT/ HR-US)	-	0 - 7.5 % (v/v)	No influence on lipase activity
Protease (free)	Borate buffer solution pH 7.5 (URT/ HR-US)	-	0 - 7.5 % (v/v)	Slight increase of protease activity at olive oil concentrations higher than 5 % (v/v)
Protease (total)	Calcium chloride solution 0.02 M (URT/ HR-US)	-	0 - 7.5 % (v/v)	No influence on total protease activity
Protease (total)	Calcium chloride solution 0.02 M (URT/ HR-US) + Triton X 100	-	0 - 7.5 % (v/v)	No influence on total protease activity
Amylase	Phosphate buffer solution (URT/ HR-US)	-	0 - 7.5 % (v/v)	Decrease of initial slopes with ascending olive oil content; high CVs above 2 % (v/v) olive oil
Amylase (Blanks)	Phosphate buffer solution (URT/ HR-US)	-	0 - 7.5 % (v/v)	Blanks showed opposed behavior: increase of slopes with ascending olive oil content
Amylase	Phosphate buffer solution 0.2 M (URT/ HR-US) (Starch in Phosphate buffer solution 0.2 M (URT/ HR-US))	-	0 - 7.5 % (v/v)	Results not evaluable due to high standard deviations
Amylase	Phosphate buffer solution 0.2 M (URT/ HR-US) (Starch in Phosphate buffer solution (URT/ HR-US))	-	0 - 7.5 % (v/v)	No influence on amylase activity
Amylase	Phosphate buffer solution 0.2 M (URT/ HR-US) + Triton X 100 (Starch in Phosphate buffer solution (URT/ HR-US))	-	0 - 7.5 % (v/v)	No influence on amylase activity
Trypsin	Triton X 100 solution (URT/ HR-US)	-	0 - 7.5 % (v/v)	Strong increase of initial slopes with ascending olive oil content (Std Work, Std FIP)
Trypsin (Blanks)	Triton X 100 solution (URT/ HR-US)	-	0 - 7.5 % (v/v)	Blanks showed same behavior: increase of slopes with ascending olive oil content; in spite of blank subtraction increase of initial slopes
Trypsin	HCl 1 mM	-	0 - 7.5 % (v/v)	No influence on trypsin activity
Trypsin	Borate buffer solution pH 7.5 (URT/ HR-US)	-	0 %/ 5 % (v/v)	Increase of initial slopes
	Phosphate buffer solution 0.2 M (Ph.Eur./ USP)	-	0 %/ 5 % (v/v)	Small increase of initial slopes, precipitation of calcium phosphate led to disturbances
	Calcium chloride solution 0.02 M (URT/ HR-US)	-	0 %/ 5 % (v/v)	No influence on trypsin activity
	Calcium chloride solution 0.02 M (URT/ HR-US) + Triton X 100	-	0 %/ 5 % (v/v)	Stronger increase of initial slopes compared to HCl/ Triton X 100

3.2.2 Precision

As precision is another important criterion for the evaluation of an analytical procedure, the repeatability of enzyme activity measurements by ultrasonic methods was investigated for amylase, lipase, protease and trypsin (Table 19). Two different methods were used of how to add the enzyme solution: On the one hand, the measurement cell was opened after baseline check again to inject the enzyme solution by using one pipette for injection and another for mixing (Cell injection method), on the other hand, the measurement cell was filled with substrate and enzyme solution simultanously using a pre-mix of both solutions (Pre-mixing method). For HR-US, the cell injection was also done by using a syringe and injecting the enzyme solution through a septum without opening the cell again after baseline check. Mostly, the use of the Pre-mixing method led to improved repeatabilites compared to the cell injection method. Using the FTS and the Pre-mixing method, best repeatabilities were observed (Working with the FTS, the Cell injection method was non-applicable due to the closed system of the FTS).

In-process material of the API process generally shows high amylase activities. As PAT methods should be fast and easy, sample preparation should be avoided (e.g. dilution prior to measurement). In order to investigate the precision when a highly concentrated sample was injected, 37.5 µl (typically 300 µl) of a 0.4 % (w/v) amylase working standard solution (eight-fold higher concentration than typically used) were injected. The repeatability was 0.89 % using HR-US FTS.

In order to evaluate the influence of fibers on the ultrasonic measurement, the repeatability for the ultrasonic measurements of free protease activity in pancreas (dispersed in water) and a suspension (after separation of fibers) was determined using HR-US FTS.

Table 19. Repeatability of enzyme activity measurements by ultrasonic methods using URT, HR-US and HR-US FTS. Ultrasonic measurements were performed after adding the working standard solution to the respective substrate preparation at 37°C. Two basically different methods were used of how to add the enzyme solution (Cell injection method via pipette/ syringe and Pre-mixing method). Working with the FTS, the Cell injection method was non-applicable. Measurements not performed are marked with a dash.

	Method of enzyme addition	URT (%)	HR-US (%)	HR-US FTS (%)
Amylase	Cell injection	1.86	* 2.28	*non-applicable*
	Premixing	** 1.30	** 1.75	** 0.67
Lipase	Cell injection	1.80	1.43	*non-applicable*
	Premixing	0.91	2.44	1.42
Protease (free)	Cell injection	*** 2.16	* 1.36	*non-applicable*
	Premixing	1.71	1.25	0.55
Protease (total)	Cell injection	-	-	*non-applicable*
	Premixing	1.45	1.48	-
Trypsin	Cell injection	1.73	* -	*non-applicable*
	Premixing	-	-	-

* Using a syringe and injection through a septum the repeatability obtained was 1.99 % for amylase, 2.13 % for free protease and 1.95 % for trypsin

** Determination in starch 3 % (w/v), other results in starch 6 % (w/v)

*** Determination in casein 0.63 % (w/v), other results in casein 1.25 % (w/v)

The samples were either filtered before measurement or directly used. The samples were injected into the casein solution without previous sample dilution. For comparison the masses of the injected volumes were determined (Table 20).

For the replications of the sample without previous fiber separation, high variations were observed. Following filtration repeatability was reassessed. Evaluation referring to the mass showed improved repeatability compared to the evaluation referring to the volume. Measuring the suspension after fiber separation the repeatability was improved compared to the pancreas dispersed in water.

In order to determine internal laboratory variations such as the performance by different operators and on different days or the use of different reagent batches, the intermediate precision of enzyme activity measurements by ultrasonic methods was investigated for amylase lipase, protease and trypsin (Table 21). Except for lipase method (HR-US), intermediate precisions were improved by using the Pre-mixing method.

Table 20. Repeatability for the determination of free protease in pancreas dispersed in water and a suspension after fiber separation using HR-US FTS. Ultrasonic measurements were performed after adding the sample to casein solution (URT/ HR-US) at 37°C. Pancreas dispersed in water was used for measurement before and after filtration.

In-process sample	Filtration	n (2 repl.)	Repeatability (%) [volume]	Repeatability (%) [mass]
Pancreas dispersed in water (Start of API process)	no	3	39.09	11.82
	yes	2	5.43	0.91
Suspension after fiber separation (Middle of API process)	no	6	1.22	2.06

Table 21. Intermediate precision of enzyme activity measurements by ultrasonic methods using URT and HR-US. Ultrasonic measurements were performed after adding the working standard solution to the respective substrate preparation at 37°C. Two basically different methods were used of how to add the enzyme solution (Cell injection method via pipette/ syringe and Pre-mixing method). Measurements not performed are marked with a dash.

	Method of enzyme addition	URT (%)	HR-US (%)
Amylase	Cell injection	4.48	-
	Premixing	**1.97	**2.21
Lipase	Cell injection	-	0.58
	Premixing	2.62	1.26
Protease (free)	Cell injection	***9.02	-
	Premixing	4.81	3.40
Protease (total)	Cell injection	-	-
	Premixing	3.08	2.09
Trypsin	Cell injection	2.64	-
	Premixing	-	-

** Determination in starch 3 % (w/v), other amylase results in starch 6 % (w/v)
*** Determination in casein 0.63 % (w/v), other free protease results in casein 1.25 % (w/v)

3.2.3 Linearity

As linearity is one important criterion for the evaluation of an analytical procedure, the linearity of enzyme activity measurements by ultrasonic methods was investigated for amylase, lipase, protease and trypsin (2.2.1.2 - 2.2.1.3, 2.2.1.5). In order to determine linearity, ultrasonic velocity change was measured by injecting different concentrations of working standard solution. Depending on the concentration of enzyme typically used, different ranges of enzyme concentration were investigated for amylase, lipase, protease and trypsin. Although a broad linear range was observed for all enzymes, no proportionality was found (no line through orign). As an example, results of lipase determination are shown in Figure 22. Linearity was confirmed in the range between 0.2 and 0.5 % (w/v) concentrated working standard solution. Concentrations of 0.1 and 0.6 % (w/v) were not within the linear range. Tested ranges and linear ranges for amylase, protease and trypsin are presented in Table 22 and **Appendix A X**.

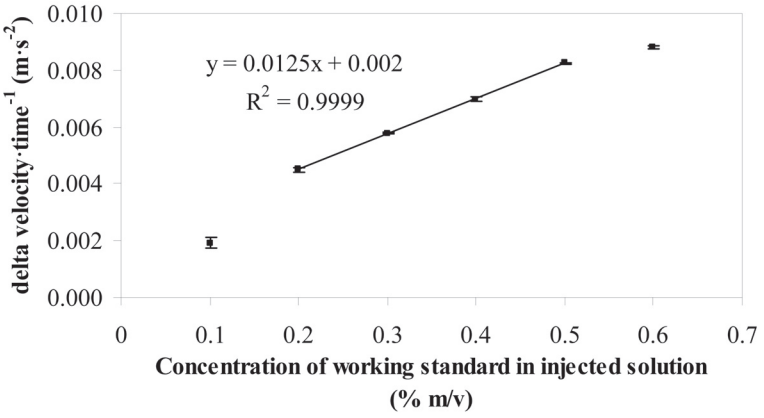

Figure 22. Test of linearity of lipase activity measurement by ultrasonic method (HR-US). Lipase working standard solutions (20 µl) with different concentrations in the range 0.1 to 0.6 % (w/v) were injected into 1050 µl of final olive oil emulsion (URT/ HR-US). Ultrasonic measurements were carried out at 37°C. Linearity was observed in the range between 0.2 and 0.5 % (w/v) working standard solution. Shown are means +/- stdev (n = 2 (2 replicates)) to improve clarity of the figure.

Table 22. Linearity experiments of amylase, protease and trypsin activity measurement by ultrasonic method (HR-US, FTS or URT). Ultrasonic measurements were performed after adding the sample solution to the respective substrate preparation at 37°C. Work Std means working standard solution prepared in Phosphate buffer solution (URT/ HR-US) or Borate buffer solution pH 7.5 (URT/ HR-US) and Triton X 100 solution (URT/ HR-US), respectively; Drug product I stands for Drug product I solution prepared in Calcium chloride 0.02 M (URT/ HR-US). FIP Std means Trypsin FIP reference standard solution prepared in Triton X 100 solution (URT/ HR-US).

		Enzyme concentration			Technique
		According to developed method (2.1.3)	**Tested range**	**Linear range**	
Amylase	**Starch 6 % (w/v)**	0.05 % (w/v) Work Std	0.01 - 0.2 % (w/v) Work Std	0.01 - 0.2 (w/v) Work Std	HR-US
	Starch 3 % (w/v)	0.05 % (w/v) Work Std	0.01 - 0.15 % (w/v) Work Std	0.01 - 0.1 (w/v) Work Std	HR-US FTS
Protease (free)		0.5 % (w/v) Work Std	0.1 - 1.5 % (w/v) Work Std	0.1 - 1.1 % (w/v) Work Std	HR-US and FTS
Protease (total)		1.8 % (w/v) Drug product I	0.6 - 3.0 % (w/v) Drug product I	0.6 - 3.0 % (w/v) Drug product I	URT
Trypsin		1.0 % (w/v) Work Std	0.8 - 1.2 % (w/v) Work Std	0.8 - 1.2 % (w/v) Work Std	URT
		0.03 % (w/v) FIP Std	0.03 - 0.04 % (w/v) FIP Std	0.03 - 0.04 % (w/v) FIP Std	URT

3.2.4 Robustness

3.2.4.1 Influence of temperature

As ultrasonic velocity shows a strong dependency on temperature, the influence of small temperature differences (36.8 - 37.2°C) was investigated to estimate the impact of temperature decreases caused by enzyme injection. Due to the differences in system tempering, the test was performed for URT as well as HR-US. No influences were observed for all enzymes (**Appendix A XI**).

Additionally, wide temperature ranges (25 - 37°C) were tested to reproduce information about properties of pancreatic enzymes from the literature. Comparable behavior of the pancreatic enzymes towards temperature between ultrasonic analysis and literature indicate the monitoring of enzyme activity. No increase of amylase activity was observed from 25 to 30°C, using 1 % (w/v) starch solution and 0.1 % (w/v) working standard solution. Using a 6 % (w/v) starch solution, amylase activity increased with ascendig temperature over the whole temperature range tested, in agreement with the literature (Ruyssen & Lauwers, 1978). Lipase, protease and trypsin activity also increased with ascending temperature in the tested range (**Appendix A XII**).

3.2.4.2 Influence of substrate concentration

Since a suitable substrate concentration is mandatory to reach a linear dependency between ultrasonic signal and enzyme concentration, different concentrations of substrate (starch, olive oil, casein and BAEE) were introduced for the ultrasonic determination of amylase, lipase, protease and trypsin activity.

The change of ultrasonic velocity decreased with ascending casein or BAEE solution regarding the determination of protease and trypsin activity (Table 23, **Appendix A XIII**).

Table 23. Influence of substrate concentration on ultrasonic determination of pancreatic enzymes. Solutions/ Emulsions with different conent of substrates were prepared. Ultrasonic measurements were performed after adding the working standard solution to the respective substrate preparation at 37°C.

	Substrate	Substrate concentration		Result
		According to developed method (2.1.3)	Tested range	
Amylase	Starch	3 %/ 6 % (w/v)	1 - 8 % (w/v)	No influence on ultrasonic velocity change using starch solution 2 - 8 % (w/v)
Lipase	Olive oil	3 % (v/v)	2 - 5 % (v/v)	No influence on ultrasonic velocity change
Protease (free)	Casein	1.25 % (w/v)	0.3 - 1.3 % (w/v)	No influence on ultrasonic velocity change
Protease (total)	Casein	1.25 % (w/v)	0.6 - 2.5 % (w/v)	Decrease of ultrasonic velocity change with ascending casein concentration; plateau in the range between 1.3 and 1.9 % (w/v)
Trypsin	BAEE	0.2 % (w/v)	0.1 - 0.4 % (w/v)	Slight decrease of ultrasonic velocity change with ascending BAEE concentration within limits +/- 3 s

3.2.4.3 Stability of substrate preparation over time

According to pharmacopoeias, fresh substrate preparations need to be prepared on the day of analysis. As preparation of substrate solutions is time-consuming and only very small amounts of substrate solutions are used for one ultrasonic analysis, stability of substrate preparations was tested over several days. Therefore, enzyme activity in freshly prepared substrate solution and emulsion, respectively, was determined. Defined time intervals later the measurements were repeated using the same substrate preparation. The olive oil preparation was stored at 2 - 8 °C. Other substrate solutions were stored at room temperature. Starch and casein solutions showed turbidity after 48 hours and the ultrasonic measurements showed high CVs. The BAEE solution and the olive oil emulsion were stable within the tested ranges of 48 hours and 21 days, respectively (Table 24, **Appendix A XIV**).

Table 24. Test of substrate stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of pancreatic enzyme activities. Amylase, lipase, protease and trypsin activities in freshly prepared substrate solution and emulsion, respectively, were determined. Defined time intervals later the measurements were repeated using the same substrate preparation (olive oil preparation was stored at 2 - 8 °C, other preparations were stored at room temperature).

	Substrate	Storage time of substrate preparation		Result
		According to developed method (2.1.3)	Tested range	
Amylase	Starch 6 % (v/v)	-	0 - 48 h	Stable for 24 h (high CV after 48 h)
Lipase	Olive oil	-	0 - 21 d	Stable for 21 d
Protease (free)	Casein	-	0 - 48 h	Stable for 24 h (high CV after 48 h)
Protease (total)	Casein	-	0 - 96 h	Small decrease of activity after 96 h
Trypsin	BAEE	-	0 - 48 h	Stable for 48 h

3.2.4.4 Influence of extraction (dissolution) time

For extraction (dissolution) of pancreas powder in the sample solvent, 15 to 20 min are needed. Longer extraction times were tested in case of disturbances during routine measurements. The enzymatic activities were not influenced by extraction (dissolution) times between 10 and 85 min (**Appendix A XV**).

3.2.4.5 Stability of enzyme in titration equipment

In order to test the applicability of the titration system, enzyme stability in the titration equipment was tested for the determination of amylase activity (Figure 23 A, **Appendix A XVIII**) and free protease activity (Figure 23 B, **Appendix A XVIII**) according to 2.2.1.5 and 2.2.1.6. In particular, this equipment was conceivable to accelerate linearity experiments (3.2.3). After the enzyme solutions had been kept for a long time interval (more than 20 min) in the dosing system, losses of amylase and protease activity were observed using the titration equipment. Thus, the titration equipment was not applicable to test linearity.

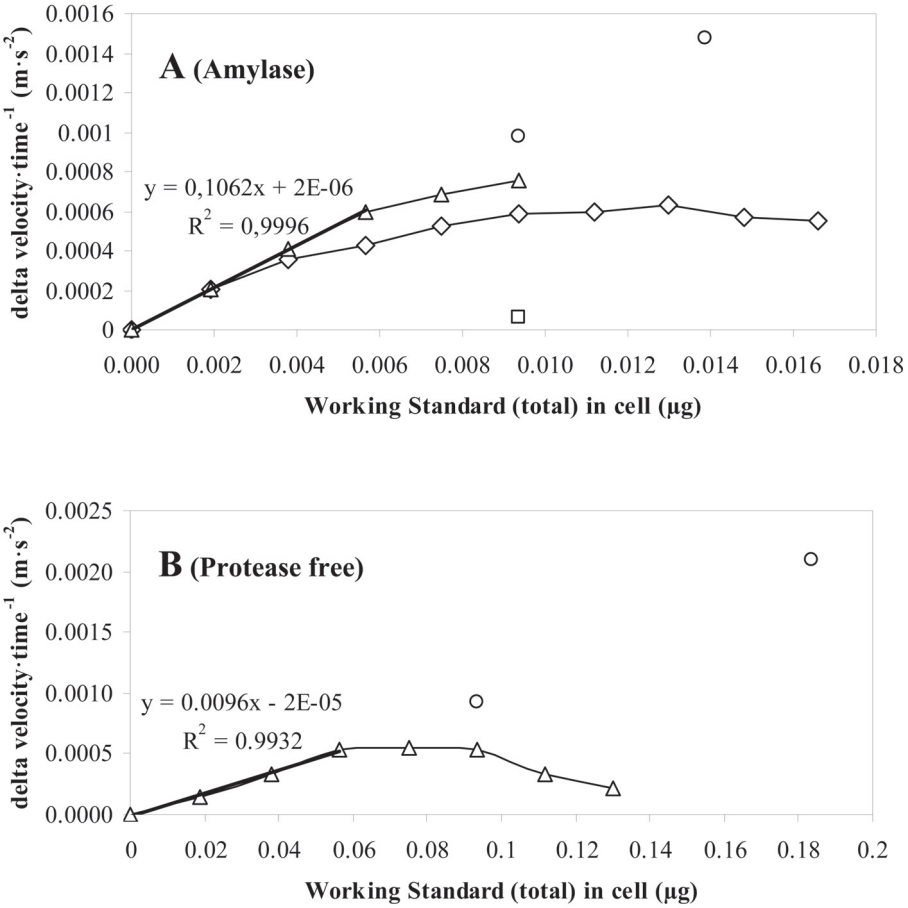

Figure 23. Test on exposure times of working standard in the titration equipment at 37 °C (HR-US). Data series marked with triangles and rhombs (Δ/ ◊) show the ultrasonic velocity changes over time after periodic injection of amylase (A) or free protease (B) solution into the cell filled with starch solution 6 % (URT/ HR-US) (A) or casein solution (URT/ HR-US) (B). The circles (○) mark data points obtained in measurements when the enzyme solution was added once after staying in the syringe for a short time interval (amylase: 20 and 30 µl, protease: 20 and 40 µl). The square (□) refers to the signal obtained when the amylase solution was added once after staying in the syringe for a long time interval (A) (n = 1 (1 replicate)).

3.2.4.6 Lipase: Potential influencing factors of lipase activity depending on substrate emulsion

Influence of bile salts in emulsion

Bile salts enhance the rate of lipolysis due to assistance in removing and solubilizing fatty acids. Based on the pharmacopoeias, an emulsion containing 6 % (w/v) bile salts (Lipase Activating Mixture Ph.Eur.) was defined. The dependency of lipase activity on the bile salts concentration was investigated. Testing a range from 0.11 to 1.05 % (w/v), concentrations above 0.4 % (w/v) showed no influence on lipase activity (**Appendix A XVI**).

Influence of pH in emulsion

As lipase activity depends on pH, the pH of the TRIS buffer was varied between 7.2 and 9.5. An increase of ultrasonic velocity change was observed between pH 7.2 to 8.4. Between pH 8.4 and 9.5 ultrasonic velocity change declined slightly (Figure 24, **Appendix A XVI**).

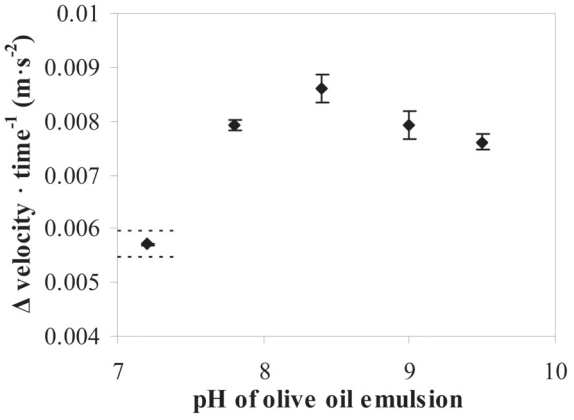

Figure 24. Influence of pH in the final olive oil emulsion on lipase activity measurement by ultrasonic method (HR-US). Olive oil emulsions and bile salts solutions were prepared with buffers of different pH values between 7.2 and 9.5. For ultrasonic measurements 20 µl of lipase solution (URT/ HR-US) were injected to 1050 µl of the final olive oil emulsions. Shown are means +/- stdev (n = 2 (2 replicates)) to improve clarity of the figure. Dashed lines represent limits of +/- 3s (s = 1.43 %).

Influence of operating time of Microfluidizer® before processing the emulsion

Since the product stream is exposed to high temperatures during processing in the Microfluidizer®, increasing with growing operating times of the homogenizer, the influence of operating time before processing the emulsion was tested. The homogenizer was run over 0, 30 and 60 min before the emulsion was processed. The experiment was replicated a second day (2. trial). The operating time showed no influence on lipase activity (**Appendix A XVI**).

3.2.4.7 Total protease: Activation parameters

In order to determine total protease activity, inactive zymogens in the sample need to be activated by adding enterokinase. Stability of the enterokinase solution (storage at 5 ± 3 °C) and the influence of enterokinase concentration, activation time and activation temperature were investigated. Change of ultrasonic velocity over time increased with ascending enterokinase concentration. This effect was eliminated by subtraction of a blank value observed by using Calcium chloride solution 0.02 M (URT/ HR-US) instead of pancreatic enzyme solution. Activation temperatures between 25 and 45 °C, activation times between 5 and 20 min and storage times up to 6 days did not influence the change of ultrasonic velocity (Table 25, **Appendix A XVII**).

Table 25. Influence of enterokinase concentration, storage (5 ± 3 °C) time of enterokinase solution and activation temperature/ time on ultrasonic determination of pancreatic enzymes. Ultrasonic measurements were performed after adding the total protease solution (URT/ HR-US) prepared under varied conditions to the casein solution (URT/ HR-US) at 37°C.

Substrate	Parameter conditions		Result
	According to developed method (2.1.3)	Tested range	
Concentration of enterokinase	1.65 % (w/v)	0.9 - 2.5 % (w/v)	No influence in concentration range between 1.3 and 2.1 % (w/v); No influence when working with blank subtraction in the tested range
Stability of enterokinase solution	0 h	0 - 72 h	No influence on activity
Activation time	15 min	0 - 20 min	No influence between 5 and 20 min
Activation temperature	35 °C	25 °C - 45 °C	No influence on activity

3.3 Amylase: Dextrins as alternative substrates

Highly concentrated starch preaparations are often unstable. In contrast to starch, an advantage of most dextrins is their ready solubility. Thus, different dextrin preparations were tested as alternative substrates to starch solution. The used dextrins differ in the content of reducing matters (Dextrin 5, 10 and 20).

The preparation of substrate solution had to be performed differently depending on the reducing matters of the dextrins. Dextrin 5 needed to be prepared following the preparation of soluble starch, whereas Dextrin 10 and 20 were readily soluble in Phosphate buffer solution (URT/ HR-US) at room temperature. The solution of Dextrin 10 and 20 were clear, the solution of Dextrin 5 showed a slight turbidity similar to starch solution.

Different dextrin concentrations were applied (5, 10, 20 % (w/v)) to ultrasonic measurements. The preparation of a 20 % (w/v) solution of Dextrin 5 was not possible. Ultrasonic velocity changes over time decreased with increasing content of reducing matters of the dextrins from Dextrin 5 to Dextrin 20. Comparing the signals obtained with Dextrin 10 and starch, same signal strengths were observed. For all investigated dextrins, the initial slopes decreased with ascending concentrations (Figure 25, **Appendix A XIX**).

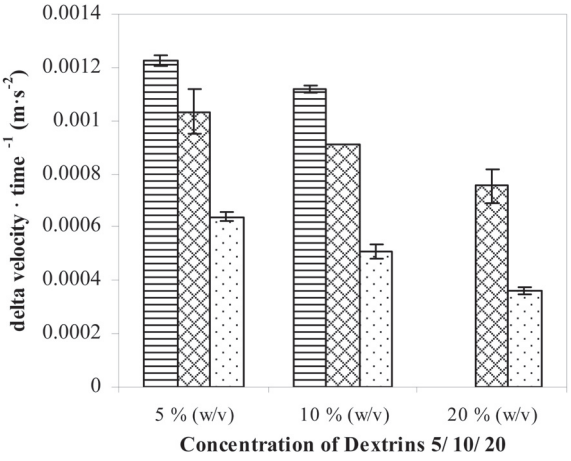

Figure 25. Suitability test of dextrins as altenative substrates for amylase activity measurement by ultrasonic method (URT). Three different concentrations (5, 10 and 20 % (w/v)) of three dextrins (striped: Dextrin 5, checkered: Dextrin 10, dotted: Dextrin 20) were tested (Preparation of a 20 % (w/v) solution of Dextrin 5 was not possible). The measurements were carried out at 37°C after injecting 4 µl of amylase solution (URT/ HR-US) into 180 µl of the dextrin solution. Shown are means +/- stdev (n = 2 (2 replicates)) to improve clarity of the figure.

3.4 Ultrasonic determination of pancreatic enzyme activities in porcine pancreas or porcine pancreas powder-containing material

3.4.1 General

Based on feasibility tests, different ultrasonic methods for the determination of amylase, lipase, protease and trypsin were designed. In order to determine enzyme activity in porcine pancreas or porcine pancreas powder-containing samples, the change of ultrasonic velocity over time was determined in sample as well as working standard solution. The relative activity was calculated using the activity label claim of the working standard (based on pharmacopoeia). Calibration was done with working standard according to 2.2.1.8. In order to verify the ultrasonic methods as alternative, comparative data were generated by determination of enzyme activity according

to pharmacopoeia (2.2.2) and ultrasonic methods (2.2.1). The use of ultrasonic methods as PAT is based on the ability to determine samples of the manufacturing process. Therefore, enzyme activities were obtained for IPC material, API and drug product (pellets).

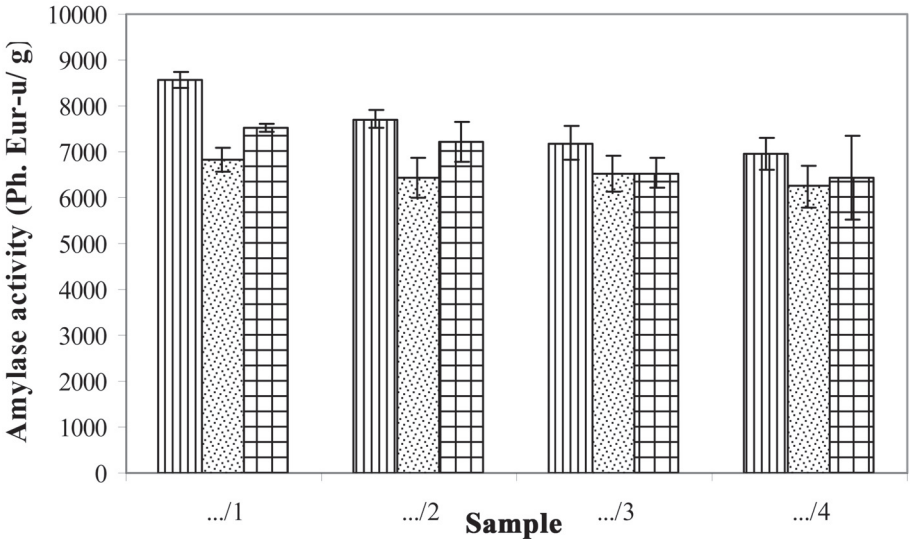

Figure 26. Relative amylase activities (FIP/ Ph.Eur.-u/g) in three batches of proteolysis-like material (striped: batch a, dotted: batch b, checkered: batch c) with four samples each were determined using URT method. The frozen samples were unfrozen and separated from fibers before measurement. 4 µl of Amylase solution (URT/ HR-US) (sample or working standard) were injected into the measurement cell, filled with 6 % starch solution (URT/ HR-US). The measurements were carried out at 37°C. Shown are means +/- stdev (n = 2 (amylase solutions with 1 replicate each) to improve clarity of the figure.

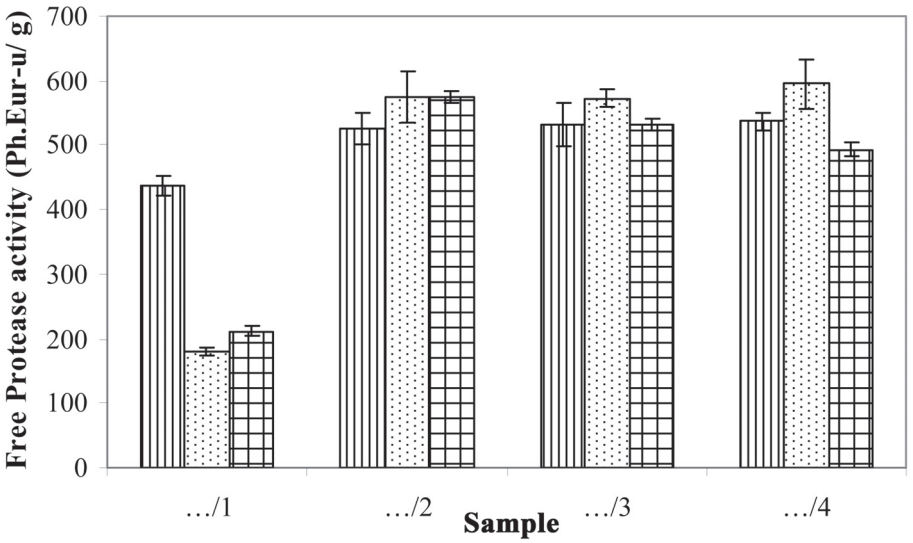

Figure 27. Relative free protease activities (FIP/ Ph.Eur.-u/g) in three batches of proteolysis-like material (striped: batch a, dotted: batch b, checkered: batch c) with four samples each were determined using URT method. The frozen samples were unfrozen and separated from fibers before measurement. 4 µl of Free protease solution (URT/ HR-US) (sample/ working standard) were injected into the measurement cell, filled with Casein solution 1.25 % (URT/ HR-US). The measurements were carried out at 37°C. Shown are means +/- stdev (n = 2 (free protease solutions with 1 replicate each) to improve clarity of the figure.

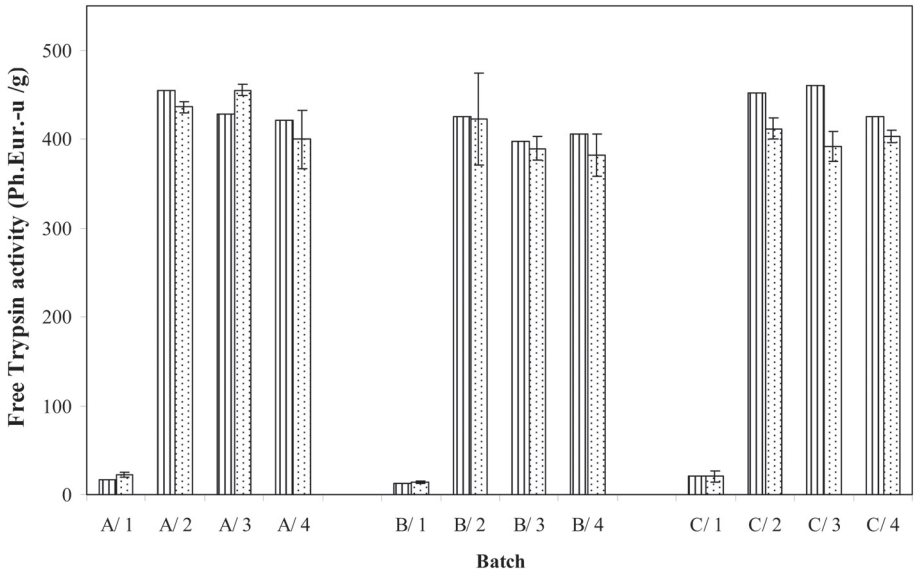

Figure 28. Relative free trypsin activities (FIP/ Ph.Eur.-u/ g) in three batches of proteolysis-like material (A, B, C) with four samples each were determined using URT method (dotted) and Ph.Eur. method (striped). For ultrasonic measurements, the frozen samples were unfrozen and separated from fibers before measurement. 4 µl of Free Trypsin solution (URT/ HR-US) (sample/ working standard) were injected into the measurement cell, filled with BAEE solution (URT/ HR-US). The measurements were carried out at 37°C.Shown are means +/- stdev (n = 2 (trypsin solutions with 1 replicate each) to improve clarity of the figure. Fresh samples were used for the Ph.Eur. method.

3.4.2 IPC material

3.4.2.1 Proteolysis-like material

Relative activities of amylase, free protease and free trypsin were determined in three batches of proteolysis-like material. For trypsin, comparative data using the pharmacopoeia method were generated. Prior to ultrasonic measurements, the free trypsin activity of the working standard was determined as 1298 U/ g using the official FIP standard (n = 5, CV = 3.1 %).

Higher values of free protease (Figure 27) and free trypsin (Figure 28) activity were observed for batch 1 compared to batches 2 - 4, whereas amylase activity was constant for all batches (Figure 26). Regarding trypsin, results of the titration method showed the same tendencies as the ultrasonic results. Mostly, lower activities were found using the ultrasonic measurement (average percentage deviation of 9 % of ultrasonic results in relation to results based on pharmacopoeia). Details are shown in **Appendix A XX**.

3.4.2.2 Proteolysis material

Proteolysis material I

Relative amylase, free protease and free trypsin activities were determined in three batches of proteolysis material (laboratory scale) using URT. Depending on the batch, amylase activities ranged from 5,000 to 11,000 FIP/ Ph.Eur.-u/ g (Figure 29, **Appendix A XXI**).

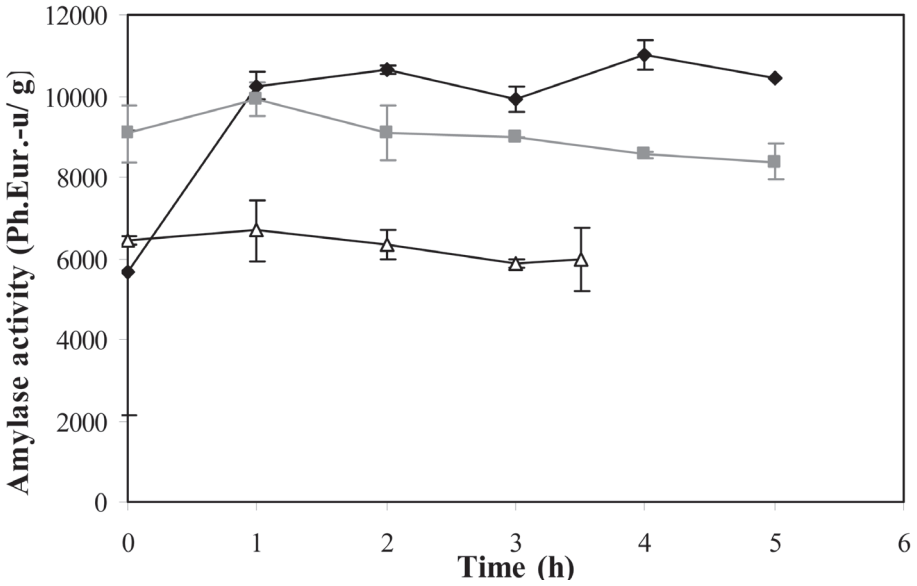

Figure 29. Relative amylase activities (FIP/ Ph.Eur.-u/g) in three batches of Proteolysis material I were determined over proteolysis time using URT method. Black/ white symboles indicate results obtained by measuring fresh samples of two batches (stored on ice for transport), grey symboles indicate results obtained by measuring frozen samples of a third batch determined two days after sampling. The samples were separated from fibers before measurement. 4 µl of Amylase solution (URT/ HR-US) (sample or working standard) were injected into the measurement cell, filled with 6 % starch solution (URT/ HR-US). The measurements were carried out at 37°C. In order to improve clarity of the figure, means +/- stdev (n = 2 (amylase solutions with 1 replicate each) are shown.

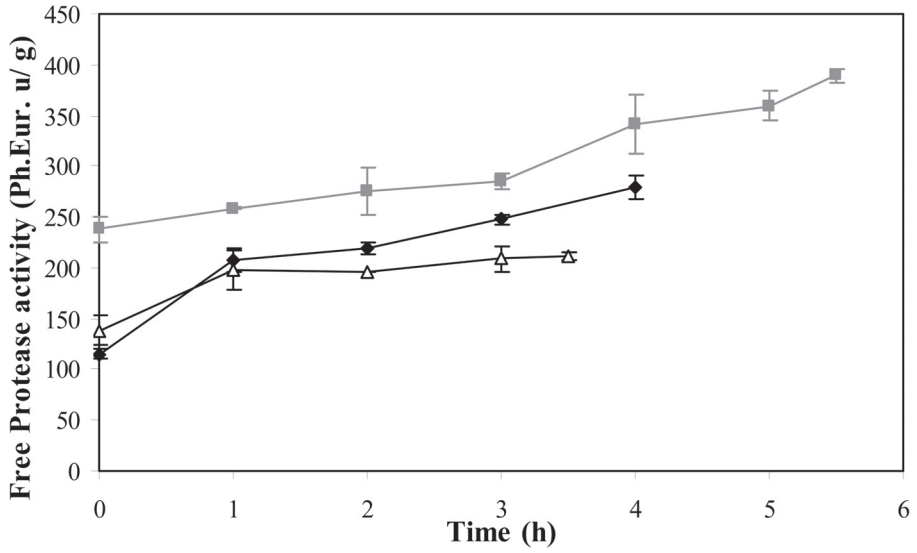

Figure 30. Relative free protease activities (FIP/ Ph.Eur.-u/g) in three batches of Proteolysis material I were determined over proteolysis time using URT method. Black/ white symboles indicate results obtained by measuring fresh samples (stored on ice for transport) of two batches, grey symboles indicate results obtained by measuring frozen samples of a third batch determined two days after sampling. The samples were separated from fibers before measurement. 4 µl of Free protease solution (URT/ HR-US) (sample or working standard) were injected into the measurement cell, filled with Casein 1.25 % solution (URT/ HR-US). The measurements were carried out at 37°C. In order to improve clarity of the figure, means +/- stdev (n = 2 (free protease solutions with 1 replicate each) are shown. [Due to instrumental disturbances the sample of the black data series was not analyzed for time 5h].

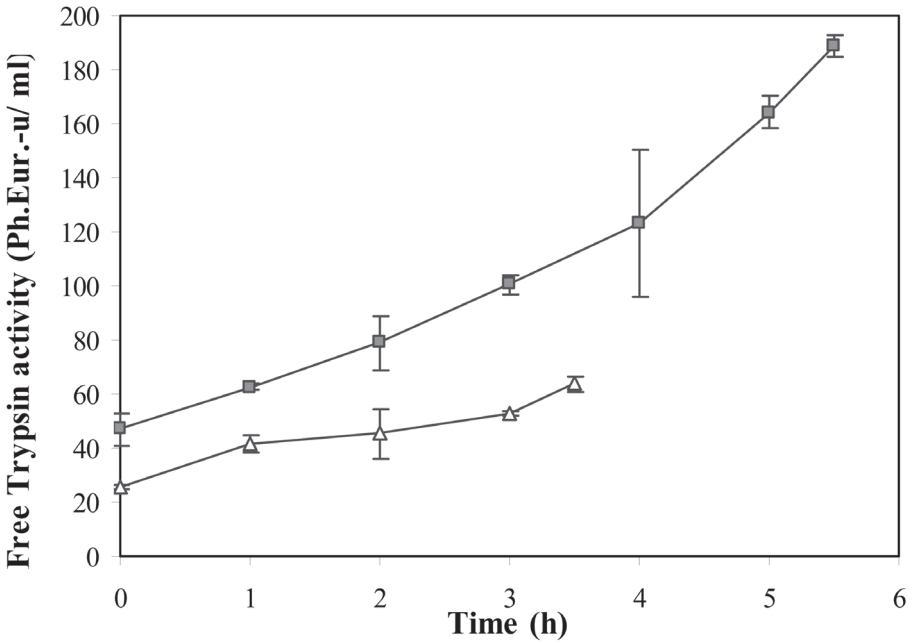

Figure 31. Relative free trypsin activities (FIP/ Ph.Eur.-u/g) in two batches of Proteolysis material I were determined over proteolysis time using URT method. The frozen samples were unfrozen and separated from fibers before measurement. 8 µl of the samples and Trypsin solution (URT/ HR-US) (FIP reference standard 42.4 u/ mg) were injected into the measurement cell, filled with BAEE solution (URT/ HR-US). The measurements were carried out at 37°C. In order to improve clarity of the figure, means +/- stdev (n = 2 (trypsin solutions with 1 replicate each) are shown.

Within a batch, constant amylase activities were observed during hydrolysis. Free protease activities increased over hydrolysis time. Free protease activities observed ranged between 100 and 400 Ph.Eur.-u/ mg (Figure 30, **Appendix A XXI**). For the determination of free trypsin activity, 8 µl of the sample were directly injected into the measurement cell, filled with BAEE solution (without previous dilution). Trypsin activity increased over hydrolysis time (Figure 31).

Proteolysis material II

Additional proteolysis samples were determined after optimizing the amylase and trypsin assays concerning fat influences on the measurement results using Phosphate buffer 0.2 M (URT/ HR-US) for the preparation of Amylase solution (URT/ HR-US) and Hydrochloric acid 1 mM for the preparation of Free trypsin solution (URT/ HR-US). Previously, free trypsin activity of the working standard was determined as 1387 U/ g (n = 3, CV = 1.0 %) using the official FIP standard and Ph.Eur. method. Furthermore, the solid content was determined for the whole hydrolysis time according to 2.2.4.

Relative amylase and lipase activities were stable (Figure 32, Figure 35), whereas relative free protease (Figure 33) and free trypsin (Figure 36) activities increased in the course of proteolysis, and total protease activity (Figure 34) decreased over time (**Appendix A XXII**). The enzyme activities showed no dependencies on the solid content (Table 26).

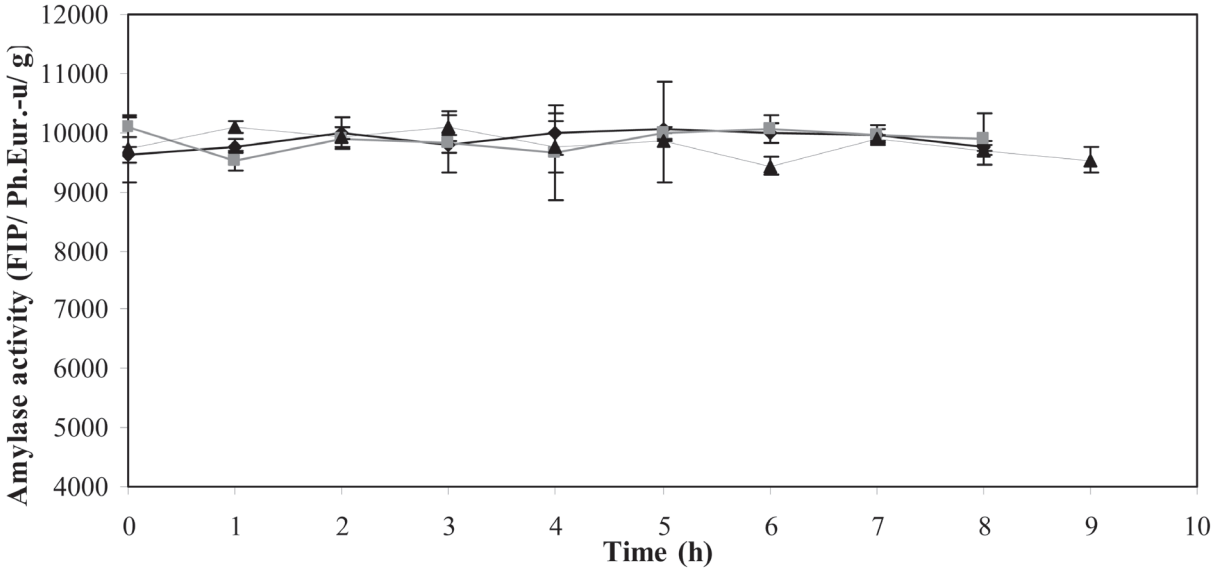

Figure 32. Relative amylase activities (FIP/ Ph.Eur.-u/g) in three batches of Proteolysis material II (batch a: rhomb, batch b: square, batch c: triangle) were determined using URT method. One batch (grey symboles) was analyzed by measuring fresh samples (stored on ice for transport) and two batches (black symboles) were analyzed by measuring unfrozen samples. Fibers were not separated. The measurement cell was filled with a pre-mix of 1800 µl Starch solution 3 % (URT/ HR-US) and 40 µl of Amylase solution (prepared in Phophate buffer solution 0.2 M). The measurements were carried out at 37°C. In order to improve clarity of the figure, means +/- stdev (n = 2 (amylase solutions with 2 replicates each) are shown.

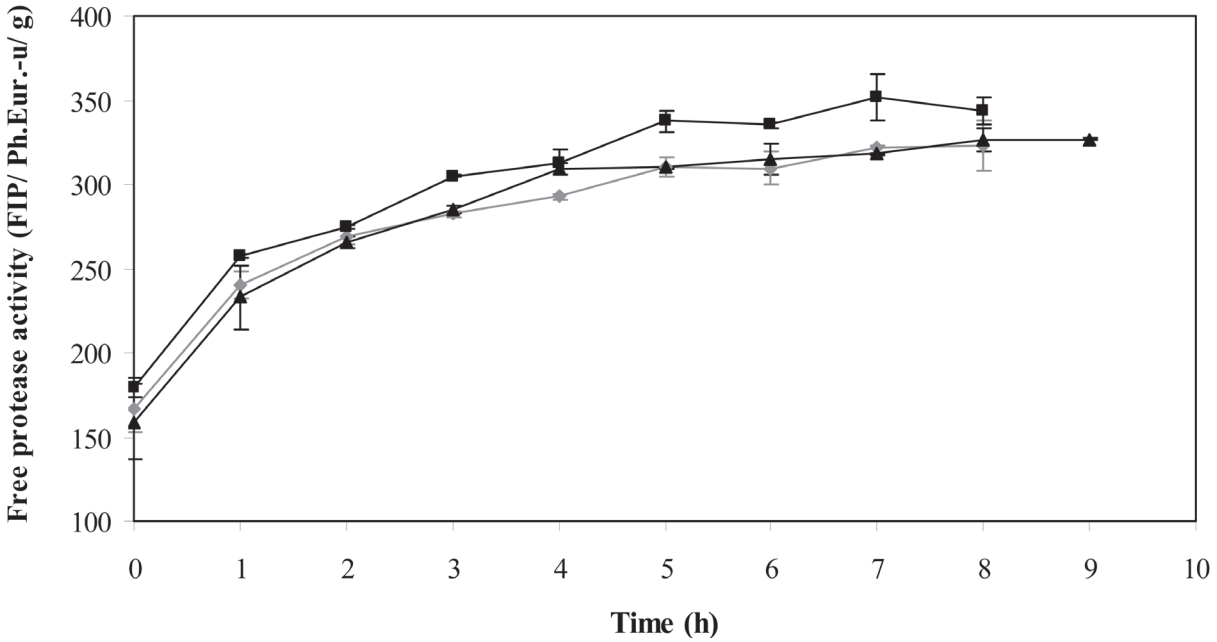

Figure 33. Relative free protease activities (FIP/ Ph.Eur.-u/g) in three batches of Proteolysis material II (batch a: rhomb, batch b: square, batch c: triangle) were determined using URT method. One batch (grey symboles) was analyzed by measuring fresh samples (stored on ice for transport) and two batches (black symboles) were analyzed by measuring unfrozen samples. Fibers were not separated. The measurement cell was filled with a pre-mix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 40 µl of Free protease solution (URT/ HR-US). The measurements were carried out at 37°C. In order to improve clarity of the figure, means +/- stdev (n = 2 (free protease solutions with 2 replicates each) are shown.

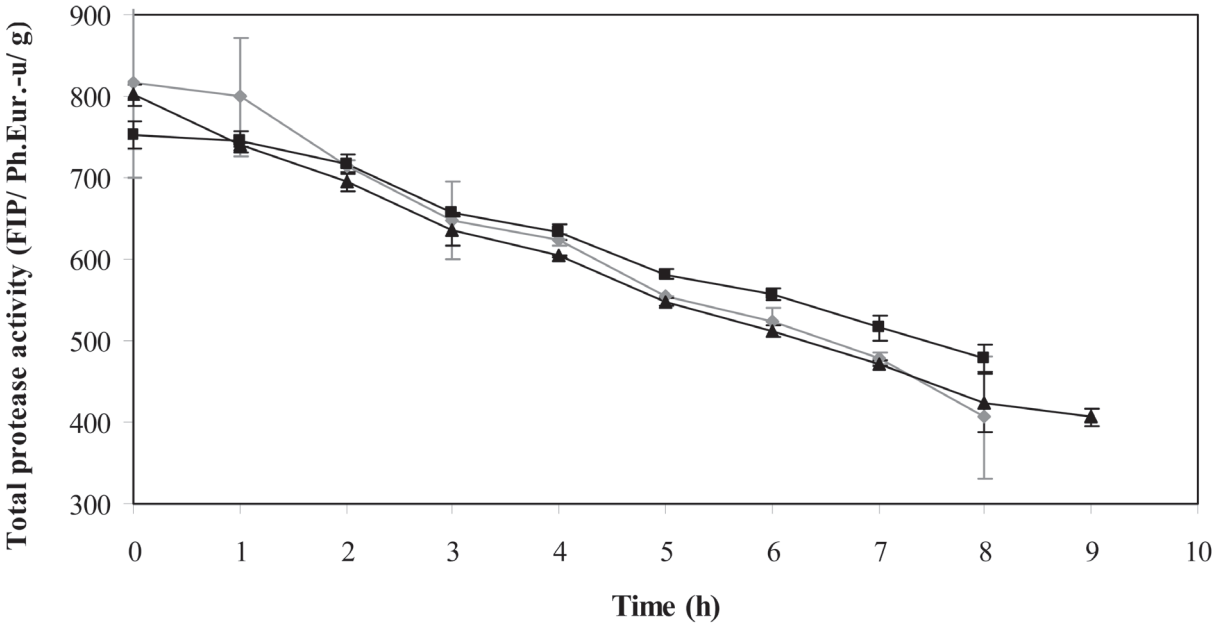

Figure 34. Relative total protease activities (FIP/ Ph.Eur.-u/g) in three batches of Proteolysis material II (batch a: rhomb, batch b: square, batch c: triangle) were determined using URT method. One batch (grey symboles) was analyzed by measuring fresh samples (stored on ice for transport) and two batches (black symboles) were analyzed by measuring unfrozen samples. Fibers were not separated. The measurement cell was filled with a pre-mix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 80 µl of activated Total protease solution (URT/ HR-US). Sample incubation was performed at 35 °C for 15 min. The ultrasonic measurements were carried out at 37°C. In order to improve clarity of the figure, means +/- stdev (n = 2 (total protease solutions with 2 replicates each) are shown.

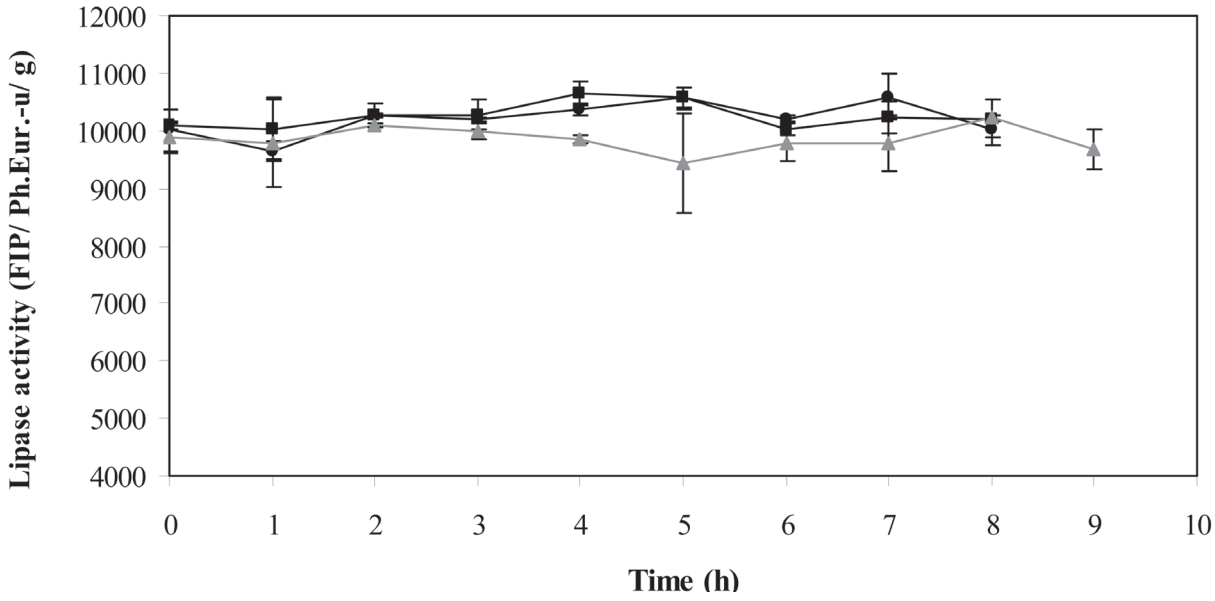

Figure 35. Relative lipase activities (FIP/ Ph.Eur.-u/g) in three batches of Proteolysis material II (batch a: rhomb, batch b: square, batch c: triangle) were determined using URT method. One batch (grey symboles) was analyzed by measuring fresh samples (stored on ice for transport) and two batches (black symboles) were analyzed by measuring unfrozen samples. Fibers were not separated. The measurement cell was filled with a pre-mix of 1800 µl Olive oil emulsion (URT/ HR-US) (final) and 33 µl of lipase solution (URT/ HR-US). The measurements were carried out at 37°C. In order to improve clarity of the figure, means +/- stdev (n = 2 (lipase solutions with 2 replicates each) are shown.

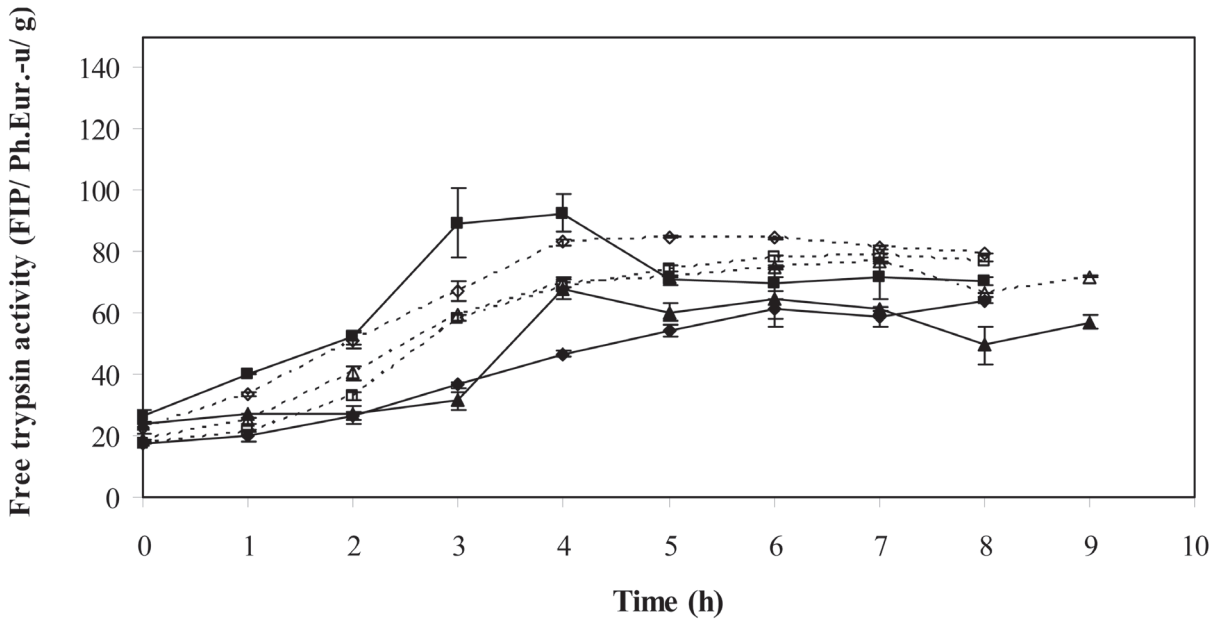

Figure 36. Relative free trypsin activities (FIP/ Ph.Eur.-u/g) in three batches of Proteolysis material II (batch a: rhomb, batch b: square, batch c: triangle) were determined using URT method. One batch (grey symboles) was analyzed by measuring fresh samples (stored on ice for transport) and two batches (black symboles) were analyzed by measuring unfrozen samples. Fibers were not separated. The measurement cell was filled with a pre-mix of 1800 µl BAEE solution (URT/ HR-US) and 80 µl of Trypsin solution (prepared in HCl 1 mM). The measurements were carried out at 37°C. The samples were also analyzed by using Pharmacopoeia method (dashed): Titrations were carried out at pH 8 and 25°C using 0.02 M NaOH as titration solution. In order to improve clarity of the figure, means +/- stdev (n = 2 (free trypsin solutions with 2 replicates each) are shown.

Table 26. Solid contents of proteolysis material (n=2)

Time/ h	Batch a		Batch b		Batch c	
	Solid content/ %	CV/ %	Solid content/ %	CV/ %	Solid content/ %	CV/ %
1	90.5	0.8	90.5	0.8	91.5	0.8
2	82.0	1.7	80.0	1.8	82.5	0.9
3	70.0	0.0	70.0	0.0	78.0	0.0
4	44.5	1.6	44.0	3.2	63.0	0.0
5	29.5	2.4	27.5	2.6	50.0	0.0
6	21.0	0.0	20.5	3.4	30.0	0.0
7	17.0	0.0	16.0	0.0	24.0	0.0
8	12.5	5.7	12.5	5.7	19.0	0.0
9	-	-	-	-	17.5	4.0

Concerning amylase, protease and lipase, reference values with the pharmacopoeia methods were determined several weeks later for selected frozen samples (proteolysis time of 3 h and 7 h) (Table 27). Lower amylase activities were observed, with up to 50 % percentage deviations of URT method results in relation to Ph.Eur. method results. Lipase activities were comparable to the results obtained with URT method. Based on measurements according to Ph.Eur., free protease activity decreased during hydrolysis whereas ultrasonic measurements showed an increase of free protease activity over time. Concerning total protease activity, both methods led to results with same tendencies, i.e. a decrease of total protease activity during hydrolysis was observed. However, the extent of decrease was different depending on the method used

(Table 27). Free trypsin activity was determined in all samples using both methods. Although the same tendencies were observed, up to 60 % percentage deviations of the URT method in relation to the Ph.Eur. method were found (Figure 36).

Table 27. Amylase, lipase, free and total protease activity were determined in selected samples of Proteolysis material II (Proteolysis 3 h, 7 h) using Ph.Eur. methods (n = 2 (1 replicate each)). Frozen samples were used for measurements which were carried out several weeks after ultrasonic measurements. Percentage deviations D (%) of URT in relation to Ph.Eur. are shown in brackets.

Proteolysis material II	**Amylase activity (Ph.Eur.-u/ g)**		**Lipase activity (Ph.Eur.-u/ g)**		**Protease (free) activity (Ph.Eur.-u/ g)**		**Protease (total) activity (Ph.Eur.-u/ g)**	
	Ph.Eur.	URT	Ph.Eur.	URT	Ph.Eur.	URT	Ph.Eur.	URT
Batch a	frozen	frozen	frozen	frozen	frozen	fresh	frozen	fresh
3 h	7,074	9,806 [38.6 %]	9,563	10,193 [6.6 %]	416	282 [32.2 %]	536	647 [20.7 %]
7 h	6,663	9,953 [49.4 %]	9,871	10,595 [7.3 %]	369	322 [12.7 %]	439	479 [9.1 %]
Batch b	frozen	fresh	frozen	frozen	frozen	frozen	frozen	frozen
3 h	7,199	9,840 [36.7 %]	9,383	10,275 [2.2 %]	441	305 [30.8 %]	529	657 [24.2 %]
7 h	6,568	9,970 [51.8 %]	9,079	10,237 [3.4 %]	365	351 [3.7 %]	440	516 [17.3 %]
Batch c	frozen	frozen	frozen	fresh	frozen	frozen	frozen	frozen
3 h	6,939	10,078 [45.2 %]	10,051	9,989 [6.5 %]	402	285 [29.1 %]	525	635 [21.0 %]
7 h	6,795	9,900 [45.7 %]	9,898	9,789 [7.8 %]	355	318 [10.4 %]	439	472 [7.4 %]

3.4.3 Active Pharmaceutical Ingredient

Relative activities of amylase, protease and lipase were determined in several API samples doing calibration by using one-point and three-point method (2.2.1.7). The results calculated using three-point calibration showed lower percentage deviations of URT in relation to Ph.Eur. (= 100 %) as compared to the results calculated using one-point calibration. Furthermore, one-point calibration often resulted in lower values determined by ultrasonic methods compared to the pharmacopoeia methods, whereas no tendency was observed for the three-point calibration (Table 28, Table 29, **Appendix A XXIII**). Concerning amylase activity, a high percentage deviation of URT in relation to Ph.Eur. was observed for Sample a. The value was confirmed measuring the sample again.

Table 28. Amylase, Lipase and free and total Protease activity were determined in API using ultrasonic methods (URT or HR-US) and three-point calibration. Ultrasonic measurements concerning lipase and free protease were carried out by using the cell-injection method, whereas the pre-mix method was performed concerning the determination of amylase and total protease activities (n = 2 (2 replicates each)). The measurements were carried out at 37°C. The samples were also analyzed by using Ph.Eur. methods (n = 2 (2 replicates each)). Percentage deviations D (%) of URT in relation to Ph.Eur. are shown in parentheses.

API: Dried material	Amylase activity (Ph.Eur.-u/ g)		Lipase activity (Ph.Eur.-u/ g)		Protease (free) activity (Ph.Eur.-u/ g)		Protease (total) activity (Ph.Eur.-u/ g)	
	Ph.Eur.	URT	Ph.Eur.	HR-US	Ph.Eur.	HR-US	Ph.Eur.	HR-US
Sample a	107,141	93,851 [12.4%]	101,460	100,561 [0.9%]	4,811	4,738 [1.5%]	5,843	5,725 [2.0%]
Sample b	109,440	107,968 [1.4%]	100,266	102,911 [2.6%]	4,839	4,938 [2.1%]	5,808	6,208 [6.9%]
Sample c	109,298	107,317 [1.8%]	101,767	103,250 [1.5%]	4,830	4,898 [1.4%]	5,858	5,931 [1.3%]
Sample d	105,301	102,707 [2.5%]	95,614	98,890 [3.4%]	4,838	4,753 [1.8%]	6,111	5,906 [3.4%]
Sample e	104,173	101,411 [2.7%]	96,238	96,473 [0.2%]	4,783	4,692 [1.9%]	5,966	5,849 [2.0%]
Sample f	102,951	100,547 [2.3%]	95,025	96,409 [1.5%]	4,792	4,651 [2.9%]	5,803	5,950 [2.5%]
Sample g	101,670	99,463 [2.2%]	93,799	96,888 [3.3%]	4,911	5,090 [3.6%]	5,901	5,902 [0.0%]
Sample h	100,909	99,895 [1.0%]	92,640	92,682 [0.1%]	4,919	4,754 [3.3%]	6,046	5,830 [3.6%]

Table 29. Amylase, Lipase, free and total Protease activity were determined in API using ultrasonic methods (URT or HR-US) and one-point calibration. Ultrasonic measurements concerning lipase and free protease were carried out by using the cell-injection method, whereas the pre-mix method was performed concerning the determination of amylase and total protease activities (n = 2 (2 replicates each)). The measurements were carried out at 37°C. The samples were also analyzed by using Ph.Eur. methods (n = 2 (2 replicates each)). Percentage deviations D (%) of URT in relation to Ph.Eur. are shown in parentheses.

API: Dried material	Amylase activity (Ph.Eur.-u/ g)		Lipase activity (Ph.Eur.-u/ g)		Protease (free) activity (Ph.Eur.-u/ g)		Protease (total) activity (Ph.Eur.-u/ g)	
	Ph.Eur.	URT	Ph.Eur.	HR-US	Ph.Eur.	HR-US	Ph.Eur.	HR-US
Sample a	107,141	92,920 [13.3%]	101,460	98,924 [2.5%]	4,811	4,518 [6.1%]	5,843	5,740 [1.8%]
Sample b	109,440	105,159 [3.9%]	100,266	99,974 [0.3%]	4,839	4,698 [2.9%]	5,808	6,328 [9.0%]
Sample c	109,298	104,576 [4.3%]	101,767	101,075 [0.7%]	4,830	4,778 [1.1%]	5,858	5,992 [2.2%]
Sample d	105,301	100,594 [4.5%]	95,614	96,608 [1.0%]	4,838	4,652 [3.8%]	6,111	5,950 [2.6%]
Sample e	104,173	99,235 [4.7%]	96,238	94,547 [1.8%]	4,783	4,589 [4.1%]	5,966	5,888 [1.3%]
Sample f	102,951	98,736 [4.1%]	95,025	94,436 [0.6%]	4,792	4,551 [5.0%]	5,803	6,008 [3.5%]
Sample g	101,670	98,998 [2.6%]	93,799	95,005 [1.3%]	4,911	4,954 [0.9%]	5,901	5950 [0.8%]
Sample h	100,909	98,072 [2.8%]	92,640	92,528 [0.1%]	4,919	4,650 [5.5%]	6,046	5,856 [3.1%]

3.4.4 Drug product

3.4.4.1 Drug product II

An inter-laboratory comparison was performed between five laboratories adopting the USP method for determination of relative amylase, free protease and lipase activities in Drug product II. For comparison, ultrasonic measurements of amylase and free protease activity were done in one laboratory. For practical reasons, lipase activity measurements using ultrasonic method could not be included at the same time. They were accomplished later in one laboratory, independently of the inter-laboratory comparison. Since URT measurements were carried out by one operator, only activities determined by the first operator within the inter-laboratory trial are shown concerning the determination of amylase and free protease activity. The results were highly comparable with the USP methods (Table 30 - 32).

Table 30. Amylase activity in Drug product II was determined within an inter-laboratory trial according to USP. For comparison with URT method, only activities obtained by the first operator were included. For URT method, Starch and Dextrin 10 were used as substrate. 4 µl of Amylase solution (URT/ HR-US) were injected into the measurement cell, filled with Starch solution 6 % (URT/ HR-US) or Dextrin 10 solution 10 %. The measurements were carried out at 37°C. Shown are average, standard deviation (stdev) and coefficient of variation (CV) (n = 6, 2 replicates each for USP and URT method).

	USP method					URT method	
	Lab 1	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Starch**	**Dextrin 10**
Activity (USP-u/ g)	265,781	269,122	261,276	252,697	242,952	258,102	265,105
	259,730	266,669	256,993	253,400	250,938	250,426	257,462
	275,202	272,884	265,090	254,108	250,833	249,316	269,426
	233,868	303,131	265,753	256,595	250,334	249,843	242,158
	244,435	343,358	268,879	256,802	251,027	250,125	252,161
	252,287	284,752	267,024	256,618	240,993	248,315	244,828
Average (USP-u/ g)	**255,217**	**289,986**	**264,169**	**255,037**	**247,846**	**251,021**	**255,190**
Stdev (USP-u/ g)	14,913	29,395	4,326	1,847	4,598	3,548	10,886
CV(%)	**5.8**	**10.1**	**1.6**	**0.7**	**1.9**	**1.4**	**4.3**

Table 31. Free protease activity in Drug product II was determined within a inter-laboratory trial according to USP. For comparison with URT method, only activities obtained by the first operator were included. For URT method, 4 µl of Free protease solution (URT/ HR-US) were injected into the measurement cell, filled with Casein solution 1.25 % (URT/ HR-US). The measurements were carried out at 37°C. Shown are average, standard deviation (stdev) and coefficient of variation (CV) (n = 6, 2 replicates each for USP and URT method).

	USP method					URT
	Lab 1	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**method**
Activity (USP-u/ g)	165,786	180,956	181,282	174,809	171,515	161,583
	177,169	187,443	167,431	165,547	158,809	160,730
	180,277	178,074	169,993	177,709	173,991	163,779
	155,709	189,506	166,695	159,441	152,894	169,097
	174,661	186,311	167,508	165,680	149,208	168,602
	168,304	188,937	171,504	161,836	175,820	164,997
Average (USP-u/ g)	**170,318**	**185,205**	**170,736**	**167,504**	**163,706**	**164,798**
Stdev (USP-u/ g)	8,978	4,638	5,478	7,236	11,529	3,490
CV (%)	**5.3**	**2.5**	**3.2**	**4.3**	**7.0**	**2.1**

	USP method	URT method
Activity (USP-u/ g)	55,082	54,023
	54,443	54,459
	55,884	53,814
	54,115	53,886
	54,709	52,627
	55,255	52,273
Average (USP-u/ g)	**54,915**	**53,513**
Stdev (USP-u/ g)	631	861
CV (%)	**1.2**	**1.6**

Table 32. Lipase activity in Drug product II was determined according to USP and URT method. For ultrasonic measurements, a premix of 33 µl of Lipase solution (URT/ HR-US) and 1800 µl of final olive oil emulsion (URT/ HR-US) were injected into the measurement cell. The measurements were carried out at 37°C. Shown are average, standard deviation (stdev) and coefficient of variation (CV) (n = 6, 2 replicates each for USP and URT method).

3.4.4.2 Drug product III - VIII

Relative enzyme activities were determined in additional pancreas powder-containing products (Products III - VIII) with ultrasonic methods and according to Ph.Eur. (**Appendix A XXIV)**. The results using URT, HR-US or FTS were highly comparable to the pharmacopoeia methods (median of percentage deviation D: 2.3 % (amylase), 1.6 - 1.8 % (lipase), 2.9 - 3.1 % (free protease), 2.7 % (total protease); Table 33 - 36), except for sample VII concerning amylase activity. In order to determine total protease activity by HR-US FTS, a 2.5 % (w/v) enterokinase solution was used for ultrasonic measurements. They were repeated introducing 1.65 % enterokinase solution (URT), based on feasibility studies. Higher percentage deviations of URT results in relation to Ph.Eur. method results were observed in comparison to the initial FTS experiment using the 2.5 % (w/v) enterokinase solution (Table 36).

Table 33. Relative amylase activities in Drug product III - VIII were determined according to Ph.Eur. and HR-US/ FTS method. For HR-US measurement, 20 µl of Amylase solution (URT/ HR-US) were injected into the measurement cell filled with 1050 µl of Starch solution 6 % (URT/ HR-US). For FTS measurement, the flow-through cell was filled with a pre-mix of 300 µl of Amylase solution (URT/ HR-US) and 15.75 µl of Starch solution 3 % (URT/ HR-US). The measurements were carried out at 37°C (n = 2 (2 replicates each)). D (%) indicates the percentage deviation of URT results in relation to results obtained with Ph.Eur. method.

	Ph.Eur.	HR-US		HR-US FTS	
Drug product	Amylase activity (Ph.Eur.-u/ g)	Amylase activity (Ph.Eur.-u/ g)	D (%)	Amylase activity (Ph.Eur.-u/ g)	D (%)
III	42,843	41,490	**3.2**	-	**-**
IV	42,471	41,490	**2.3**	-	**-**
V	43,763	44,072	**0.7**	44,816	**2.4**
VI	44,028	43,349	**1.5**	44,010	**0.0**
VII	44,199	40,378	**8.6**	44,598	**0.9**
VIII	45,335	-	**-**	44,261	**2.4**

Table 34. Relative lipase activities in Drug product III - VIII were determined according to Ph.Eur. and HR-US method. For HR-US measurement, 20 µl of Lipase solution (URT/ HR-US) were injected into the measurement cell filled with 1050 µl of final olive oil emulsion (URT/ HR-US). The measurements were carried out at 37°C (n = 2 (2 replicates each)). D (%) indicates the percentage deviation of URT results in relation to results obtained with Ph.Eur. method. FTS measurements were not performed.

	Ph.Eur.	**HR-US**		**HR-US FTS**	
Drug product	Lipase activity (Ph.Eur.-u/ g)	Lipase activity (Ph.Eur.-u/ g)	**D (%)**	Lipase activity (Ph.Eur.-u/ g)	**D (%)**
III	61,786	60,781	**1.6**	Not performed	
IV	60,164	60,776	**1.0**		
V	49,795	48,676	**2.3**		
VI	49,395	47,938	**3.0**		
VII	50,297	49,402	**1.8**		
VIII	55,641	55,287	**0.6**		

Table 35. Relative free protease activities in Drug product III - VIII were determined according to Ph.Eur. and HR-US/ FTS method. For HR-US measurement, 20 µl of Free protease solution (URT/ HR-US) were injected into the measurement cell filled with 1050 µl of Casein solution 1.25 % (URT/ HR-US). For FTS measurement, the flow-through cell was filled with a pre-mix of 300 µl of Free protease solution (URT/ HR-US) and 15.75 µl of Casein solution 1.25 % (URT/ HR-US). The measurements were carried out at 37°C (n = 2 (2 replicates each)). D (%) indicates the percentage deviation of URT results in relation to results obtained with Ph.Eur. method.

	Ph.Eur.	**HR-US**		**HR-US FTS**	
Drug product	Free protease activity (Ph.Eur.-u/ g)	Free protease activity (Ph.Eur.-u/ g)	**D (%)**	Free protease activity (Ph.Eur.-u/ g)	**D (%)**
III	2,502	2,434	**2.7**	Not performed	
IV	2,512	2,410	**4.1**		
V	2,620	2,452	**6.4**		
VI	2,522	2,526	**0.2**		
VII	2,547	2,478	**2.7**		
VIII	2,666	Not performed		2,628	**1.4**

Table 36. Relative total protease activities in Drug product III - VIII were determined according to Ph.Eur. and URT/ HR-US FTS method. For FTS measurement, the flow-through cell was filled with a pre-mix of 600 µl of Total protease solution (URT/ HR-US) activated with Enterokinase solution 2.5 % and 15.75 ml of Casein solution 1.25 % (URT/ HR-US). For HR-US measurement, the flow-through cell was filled with a pre-mix of 80 µl of Total protease solution (URT/ HR-US) activated with Enterokinase solution 1.65 % and 1800 µl of Casein solution 1.25 % (URT/ HR-US). The measurements were carried out at 37°C (n = 2 (2 replicates each)). D (%) indicates the percentage deviation of URT results in relation to results obtained with Ph.Eur. method.

	Ph.Eur.	**HR-US** (1.65 % EK)		**HR-US FTS** (2.5 % EK)	
Drug product	Total protease activity (Ph.Eur.-u/ g)	Total protease activity (Ph.Eur.-u/ g)	**D (%)**	Total protease activity (Ph.Eur.-u/ g)	**D (%)**
III	3105	3054	**1.6**	3245	**4.5**
IV	3057	2962	**3.1**	3144	**2.8**
V	2972	2798	**5.8**	3057	**2.9**
VI	3026	2903	**4.1**	3066	**1.3**
VII	3107	2881	**7.3**	3099	**0.3**
VIII	3100	2989	**3.6**	3134	**1.1**

4. DISCUSSION

4.1 Ultrasonic velocity measurements are applicable to determine relative enzyme activities using the pharmacopoeia declaration of the reference standard

Absolute enzyme activity describes the converted amount of substrate per time and is given in µmol/ min. This enzyme activity strongly depends on the assay design and condition. Therefore, pharmacopoeias define Ph.Eur.-units and USP-units in relation to the conditions which are described in the corresponding monographs. In addition, the pharmacopoeias stipulate the use of official USP or FIP standards with defined activities (given in FIP/ Ph.Eur.-u/ g or USP-u/ g) to calculate relative enzyme activities. While absolute enzyme activities can vary depending on substrate batch, operator or laboratory, the determination of relative activities eliminates intra- and inter-laboratory variations making measurements comparable. After developing and validating ultrasonic methods to determine pancreatic enzyme activity in the present thesis, they were compared to the pharmacopoeia methods by measuring pancreas powder-containing samples and reference standard and calculating relative enzyme activities in the samples with the declared activity of the reference standard based on pharmacopoeia method (Figure 37).

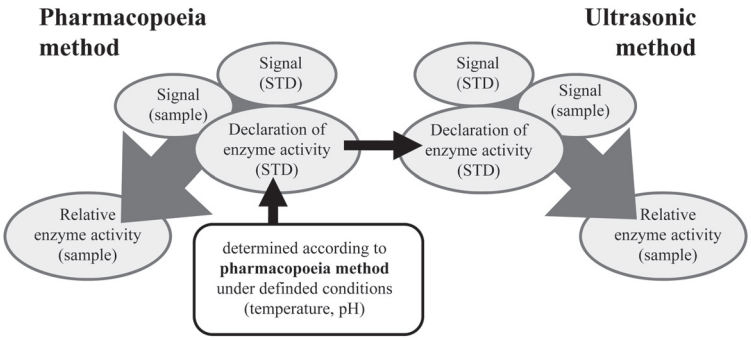

Figure 37. Evaluation of ultrasonic measurements. Relative pancreatic enzyme activity according to pharmacopoeia is calculated by relating the sample signal to the signal and the declaration (pharmacopoeia) of the reference standard. The pharmacopoeia declaration of the reference standard was also used to evaluate ultrasonic measurements after the ultrasonic velocity change of sample and reference standard had been monitored.

In order to determine absolute enzyme activities with ultrasonic methods, a correlation between substrate conversion and ultrasonic velocity would be required. According to Resa & Buckin (2011), calculations of the specific concentration increments of ultrasonic velocity a_i of substrate, product and solvent correlate ultrasonic signals and enzyme activity (1.4.4). The reported correlation model was applicable to the degradation of cellobiose to two glucose

molecules by β-glucosidase (Resa & Buckin, 2011). For this simple hydrolysis reaction, ultrasonic velocity was influenced only by one substrate (cellobiose), one product (glucose) and the solvent (water). In the case of complex substrates according to pharmacopoeias (starch, olive oil and casein), the stoichiometric relations cannot be comprehended since an endless number of intermediate substrates and products are conceivable. The unspecific ultrasonic signal of these enzyme reactions are a complex interplay of different concurrent operations such as substrate degradation to various intermediates, generation of diverse products or matrix effects of the sample. Thus, ultrasonic amylase, lipase and protease methods developed in the present thesis allow only the determination of relative enzyme activities using a reference standard. In contrast, the correlation model according to Resa & Buckin (2011) may be applicable for the degradation of trypsin as BAEE is simply cleaved to ethanol and benzoylarginine (Sacks *et al.*, 1971). However, the influence of the sample matrix on ultrasonic measurement results could be relevant, especially for the ultrasonic trypsin method as high sample concentrations are required.
Even though ultrasonic and pharmacopoeia methods are not identical, the declaration of the reference standard according to pharmacopoeias was used in the context of ultrasonic methods (see 4.2). Thus, in spite of keeping the substrates the same, the conversion of results obtained with the ultrasonic methods to pharmacopoeia units may be questioned. However, this problem is not of scientifical nature, but a formal or legal problem which could be solved by definition of a new unit (e.g. URT-unit) and the remark that one pharmacopoeia unit complies with one unit newly introduced.

4.2 Do the substrates and their preparations conform with the demands of the pharmacopoeia?

Concerning pancreas powder, several monographs exist in the pharmacopoeias. Ph.Eur. and USP give different definitions of one enzyme activity unit (USP-unit, Ph.Eur.-unit) and variations in the analysis methods for the three categories of enzymes (Creon International Scientific Brochure, 2007). Thus, the enzyme activity units are closely related to the analysis method and the conditions described in the pharmacopoeia monographs. If all conditions which influence pancreatic enzyme activities, such as substrate preparations, ionic environment, concentration ratios between substrate and enzyme, analysis time, temperature or product removal had been kept as in the pharmacopoeia monographs, identical enzyme activities would have been expected in the ultrasonic measurement cell. However, the use of ultrasonic spectroscopy required certain changes to the methods to be introduced which led to deviations from the pharmacopoeia conditions. Table 37 shows the final ultrasonic methods in comparison to the pharmacopoeia methods regarding substrate preparation, enzyme solvent, pH, temperature and additional reagents. For all enzymes, the pharmacopoeia substrate was kept, and only the preparation was optimized in case this was necessary to obtain reliable results with ultrasonic methods. For instance, higher concentrations of enzyme solutions compared to pharmacopoeias had to be

applied in order to initiate a significant change of ultrasonic velocity during enzyme reaction. This requirement was inline with one aim of the present thesis which is to analyze in-process material without sample preparation. The addition of co-factors also supported an increase in ultrasonic signals by activating the enzymatic reaction. The limited solubility of substrates and instability of substrate preparations did not allow the pharmacopoeia concentration ratios between substrate and enzyme to be maintained involving supposably the loss of substrate saturation which had to be considered within feasibility tests (e.g. linearity). As another instance, pharmacopoeia methods apply pH STAT conditions for determination of lipase or trypsin activity allowing a continuous removal of the reaction products leading to an increased enzyme activity. pH STAT conditions cannot be realized for ultrasonic measurements.

Concerning protease, the 1.25 % casein solution was adopted without modification, but the substrate-enzyme-ratio was reduced but linearity was ensured (Table 22). Considering the increased enzyme concentration, conditions of zymogen activation were adjusted (Table 25). Robustness measurements concerning activation showed no influences of varied activation times (5 to 20 min) and diverse temperatures (25 to 45°C) on protease activity.

Concerning amylase, the starch concentration and the solvent were optimized. In pre-tests using the starch solution as described in the pharmacopoeias (1 %), a dependency of amylase activity on the starch concentration was observed. The activity initially increased with ascending temperature but reached a plateau at 30°C (**Appendix A XI**), although the activity optimum is at approx. 40°C (Ruyssen & Lauwers, 1978). Both experiments indicated that substrate saturation was not achieved. Consequently, higher starch concentrations were used (Table 37). Dependencies of amylase activity on the pH or the chloride content in the sample were eliminated by preparing the substrate solution in sodium chloride-containing phosphate buffer. In addition, the temperature was increased as compared to the pharmacopoeia method. A temperature of 37 °C was chosen for all enzymes and proved to be more time efficient, because chilling the system is time-consuming. Although amylase activity is temperature-dependent, the calibration with working standard eliminates this influence (relative determination). Taken together, the ultrasonic amylase assay can be considered to resemble the pharmacopoeia method closely.

A radical change has been introduced to the lipase substrate preparation. The substrate olive oil, the bile salts as well as their ratio were kept, but the substrate-enzyme-ratio was reduced. Gum acacia had to be replaced by Triton X 100 (chapter 3.1). Calcium is an ion naturally associated with gum acacia that influences lipase activity. It was added to the substrate emulsion as calcium chloride. Differences in droplet size and different interface areas, respectively, influenced the lipase activity considerably, but the calibration with working standard eliminates this influence (relative determination). The concentration of 60 mM calcium was a compromise between lack of influence on lipase activity (Figure 17) and stability of the emulsion. TRIS (300 mM) was

introduced in higher concentrations than required for lipase activity saturation (see 3.1.2) to ensure a stable pH during lipase reaction. This decision was based on pH measurements over time during lipase reaction (**Appendix IV**). Furthermore, the pH was lowered from pH 9.0 (according to Ph.Eur.) to pH 7.2 for ultrasonic measurements. This led to more stable baselines during ultrasonic measurements which was attributed to a more stable emulsion. In emulsions with pH 8 to 9, creaming effects were observed, while this was not the case for emulsions with a pH of approx. 7. This effect was only found after long storage times. Via particle size or particle size distribution measurements, an improved stability could not be verified. The use of an emulsion with pH 7.2 also led to an improved repeatability for the ultrasonic determination of lipase activity. Nevertheless, the pH optimum of lipase was at pH 8.4 under these conditions (Figure 24). As a working standard was used for relative determination, a pH of 7.2 was acceptable and ensured reliable measurements. Consequently, results observed with Ph.Eur. and ultrasonic methods are comparable as shown for the determination of liquid IPC samples (Figure 35) , API (Tables 28 + 29) and drug product (Tables 32 + 34). However, choosing the suboptimal pH led to lower sensitivity as compared to pH 9 according to pharmacopoeias, which is also indicated by a low slope of the calibration curve observed within linearity tests (Figure 22). Taken together, the analysis of diverse samples have shown, that results obtained with pharmacopoeia and ultrasonic methods are comparable due to calibration with reference standard (relative determination).

Table 37. Comparison of ultrasonic methods and pharmacopoeia methods [MF = Microfluidizer, BAEE = N-Benzoyl-L-arginine ethyl ester hydrochloride, TRIS = Tris(hydroxymethyl) aminomethane, Mal = Maleic anhydride, TCA = Trichloroacetic acid].

	Amylase		**Lipase**		**Protease**		**Trypsin**	
	HR-US/ URT	Pharma-copoeia	HR-US/ URT	Pharma-copoeia	HR-US/ URT	Pharma-copoeia	HR-US/ URT	Pharma-copoeia
Substrate preparation	Starch 3 % (6 %)	Starch 1 %	Olive oil 3 %, bile salts 0.6 %, Triton X-100 *[MF]*	Olive oil 3 %, bile salts 0.6 %, Gum acacia *[Blender]*	Casein 1.25 % ***Total protease:*** Sample activation with EK (35 °C/ 15 min)		BAEE 0.06 M	BAEE 0.02 M
Substrate solvent	Phoshpate buffer/ NaCl	Water	TRIS/ $CaCl_2$	TRIS	Water		Borate buffer	TRIS/ HCl
pH of reaction	6.8		7.2	9.0	8.0	7.5	7.8	8.0
Sample solvent	Phosphate buffer	Phosphate buffer	TRIS/ NaCl/ Mal	***Ph.Eur.:*** TRIS/ NaCl/ Mal ***USP:*** Water	***Free:*** Borate buffer ***Total:*** $CaCl_2$	***Ph.Eur.:*** Borate buffer/ $CaCl_2$ ***USP:*** Phosphate buffer	Triton X-100/ HCl	$CaCl_2$
T (°C)	37	25	37		37	***Ph.Eur.:*** 35 ***USP:*** 40	37	25
Additional reagents	-	NaCl, HCl, I_2, NaOH, H_2SO_4, NaS_2O_3	-	NaOH	-	TCA, filters	-	NaOH

4.3 Special requirements of ultrasonic spectroscopy on substrate preparations led to the investigation of alternative excipients and substrates

The use of ultrasonic spectroscopy for the determination of pancreatic enzyme activities makes high demands on the substrate preparations as the technique is very sensitive to instabilities of the preparations, especially of emulsions (1.4.5). Since lipases are enzymes that act on non-wate-soluble substrates in an aqueous environment, the substrate used for lipase determination has to be processed into an emulsion. To obtain oil-in-water emulsions, a range of surfactants can be used and processed together with the lipid and water phases with a number of different devices. In the present thesis the aim was to measure lipase activity under conditions as close as possible to the pharmacopoeia methods. Therefore, the emulsion initially used consisted of olive oil, TRIS buffer, bile salts and gum acacia. The gum acacia-containing emulsions prepared in a household blender (Ph.Eur., USP) emerged as inapplicable since baselines measured in the ultrasonic spectrometer were unstable. The adoption of a high-pressure homogenizer (Microfluidizer®) led to smaller droplet sizes and tight droplet size distributions. But adding the Reagents mixture (Ph.Eur./ USP) after high-pressure homogenization of the Olive oil stock emulsion (Ph.Eur./ USP) led to an enlargement of particle size with increasing number of passages and pressure (Figures 11 + 12). The emulsions were "over-processed" due to high energy density, a phenomenon frequently discussed in the literature. This effect was eliminated by adding Reagents mixture (Ph.Eur./ USP) before homogenization where a reduction of droplet size was observed with increasing numbers of passages (Figure 13). But addition of the lipase co-factor calcium via calcium chloride again led to emulsion instabilities (**Appendix A II**). Jafari *et al.* (2007) also observed the phenomenon of "over-processing" expressed in re-coalescence during microfluidization at pressures above 600 bar and more than two passages and suggested *inter alia* the use of sufficient surfactant which stabilizes the new interfaces rapidly. Occupation of interfaces depends on diffusion. Polysaccharides and proteins diffuse very slowly due to their high molecular weights. They are slightly surface active, but affect the formation of the interfacial film very slowly. Gum acacia needs three or four days to occupy the interfaces (Shotton & White, 1963). The elimination of "over-processing" by adding Reagents mixture (Ph.Eur./ USP) before homogenization can be attributed to glycholic acid, taurocholic acid and their derivates contained in bile salts which show emulsifying behavior due to their amphiphilic structure. In contrast to gum acacia, they occupy the interfaces quickly which leads to an optimized stabilization during processing. Furthermore, the addition of Reagents mixture (Ph.Eur./ USP) reduced the percentage of olive oil and the viscosity of the aqueous phase. Consequently, the turbulent flow within the chambers, which is essential for droplet degradation in Microfluidizers® (Schubert, 2005), was supposably formed more easily than in the Olive oil stock emulsion (Ph.Eur./ USP) with higher viscosity.

Although the baselines of these emulsions were stable, the lipase reaction curve did not follow a general enzyme reaction curve over time after adding the lipase solution (Figure 14). An

extreme decrease of ultrasonic velocity after approx. 18 minutes was observed, accompanied by a fast droplet growth. One reason for the emulsion breaking could be the degradation of the emulsifier. Gum acacia is a complex polysaccharide which consists of a mixture of highly branched arabinogalactan heteropolymers and approx. 2 % proteins (Idris *et al.*, 1998, Al-Assaf *et al.*, 2005). Proteolytic measurements showed an increase in change of ultrasonic velocity of approx. 15 % in the gum acacia-containing casein solution as compared to pure casein solution (Figure 15) which indicate the degradation of the protein fraction in gum acacia. In the literature, the proteolytic hydrolysis of gum acacia is described as incomplete (Osman *et al.*, 1993, Mahendran *et al.*, 2008). Sabah El-Kheir *et al.* (2008) described the stabilization of gum acacia as follows: Preferentially, the protein-rich high molecular mass component adsorbs on the droplet surface. The hydrophobic polypeptide chains adsorb to the surface, whereas the carbohydrate blocks repulse each other (electrostatic and steric repulsion). Consequently, the degradation of the proteins by the pancreatic proteases is considered to lead to less adsorption of the gum acacia on the surface and therefore to low stabilization of the emulsion and increasing coalescence. Another reason for the deviating lipase reaction curve over time is seen in buffering the system. The emulsion (Ph.Eur., USP) is used in a pH STAT method according to pharmacopoeia methods. In order to keep the pH stable sodium hydroxyde is continuously added. Consequently, a high percentage of buffering components is not essential in the pharmacopoeia method (approx. 1 mM TRIS in final emulsion), whereas during ultrasonic measurements released fatty acids lead to a permanent decrease of pH which can cause the breaking of the emulsion, unless sufficient buffer capacity is present. The instability towards the addition of calcium chloride (**Appendix A II**) resulting in a decrease of pH might be also attributed to an insufficient buffering system. As calcium is contained in process samples it had to be included in the emulsion. The absence of calcium in the substrate emulsion would make the results dependent on the calcium content in the analyzed samples. Calcium also minimizes the lag phase at the beginning of the lipase reaction (Brown et al., 1977). Consequently, analysis times can be reduced in the presence of calcium, which is essential for the use of ultrasonic spectroscopy as PAT method.

Taken together, emulsion composition according to pharmacopoeias was not suitable for the ultrasonic determination of lipase activity. Gum acacia was shown to be unsuitable as emulsifier since the protein content, which can interfere with pancreatic proteases, led to increased changes of ultrasonic velocity and supposably to emulsion destabilization. The deviating course of the reaction curve over time and the instability towards calcium chloride might be also eliminated by further optimization of the buffering system.

As an alternative, the nonionic emulsifier Triton X 100 was tested. Triton X 100 is an octylphenol ethoxlate that is commonly used as detergent, wetting agent or emulsifier in oil-in-water emulsions due to an Hydrophilic-lipohilic balance (HLB) of 13.4. Owed to its non-denaturing properties,

it is often used as solubilizer for proteins. In contrast to the gum acacia-stabilized emulsions, measurements of lipase activity in Triton X 100-stabilized emulsions showed a regular course over time. In order to test quality and suitability of the emulsions to determine lipase activity via ultrasonic spectroscopy, several parameters were varied. Lipase activities as well as droplet sizes were determined for the prepared emulsions (3.1.2). Modal droplet sizes were only influenced at very low numbers of passages or emulsifier concentrations. Dependencies of lipase activity on droplet sizes were not observed. Varying the olive oil concentration, lipase activity showed the typical course described by Brown (1902) (Figure 16). Opposite effects measuring higher slopes at lower substrate concentrations were previously found for the gum acacia-containing emulsions (Figure 13). Furthermore, varying concentrations of calcium chloride (Figure 17) and TRIS (**Appendix A III**) did not cause destabilization of the emulsion, as the modal droplet size was unaffected. A plateau of lipase activity was observed above concentrations of 50 mM calcium chloride (Figure 17) and 40 mM TRIS (**Appendix A III**). Calcium chloride and TRIS had to be used at least at these concentrations to avoid influences of the sample matrix.

Caused by its low molecular weight, Triton X 100 is likely to occupy the interfaces faster than the proteins contained in gum acacia. As Triton X 100 has only a small effect on the viscosity in comparison to gum acacia, the turbulent flow stream in the Microfluidizer® was enhanced. Both effects apparently led to the elimination of the disadvantages of the gum acacia-containing emulsion. The addition of bile salts solution before high-pressure homogenization was no longer necessary saving energy costs due to lower volume and consequently lower processing times in the Microfluidizer® (50 % time saving).

Alternative substrates were considered, especially concerning the determination of amylase activity. In order to measure amylase activities in highly concentrated samples, an increase of starch concentration from 1 % (w/v) to higher concentrations was necessary for ultrasonic method. When starch concentration was increased, stability of substrate preparations decreased as indicated by growing turbidity and swelling. The solutions could not be used for more than 24 h (Table 24).The instabilities were supposably boosted by the need to dissolve starch in phosphate buffer instead of water. According to pharmacopoeias, fresh starch solution prepared on the day of analysis has to be used, although preparation of the starch solution is very time-consuming and instabilities are generally observed only after several days. In order to measure amylase activities continuously within PAT, substrate stability over several days and a fast preparation would be preferable.

As most dextrins are readily soluble in water in contrast to starch, several dextrins (5, 10, 20 % of reducing matters) were tested as alternative substrates. Dextrin 5 needed to be prepared following the preparation of soluble starch solution, whereas Dextrin 10 and 20 were readily soluble in Phosphate buffer (Ph.Eur./ USP) at room temperature. The solutions of Dextrin 10

and 20 were clear, the solution of Dextrin 5 showed a slight turbidity similar to starch solution. Based on the solubility and the strength of the ultrasonic signal, Dextrin 10 is conceivable as alternative substrate (Figure 25). Differences in ultrasonic velocity occur when the hydration shells of the molecules change. Thus, major differences in ultrasonic velocity should be monitored during degradation of a substrate with high molecular weight to small single components. Thus, substrates with low molecular weight, e.g. p-nitrophenyl-maltotrioside or derivates (Wallenfels *et al.*, 1980; Winn-Deen *et al.*, 1988), seem rather inapplicable for ultrasonic measurements. Only for starch (Mörman et al., 1982), amylose (Ishikawa *et al.*, 1990), γ-cyclodextrin (Kondo *et al.* (1989), Ishikawa *et al.*, 1990) and dextrins (Hreczuk-Hirst *et al.*, 2001), a rapid degradation by porcine pancreatic amylase via multi-chain or multiple-attack mechanism mainly to maltose is described in the literature. Thus, the choice of dextrins was basically right, but standard deviations were higher than those observed with starch solution (Table 30, **Appendix A XIX**). Furthermore, a dependency of amylase activity on the substrate concentration was found (Figure 25). An explanation for the higher variability compared to the use of starch might be the fact that the 'p.a.' grade, which is rarely available, was not introduced. Due to multiple attack mechanism, γ-cyclodextrin might be another alternative substrate. The reagent grade 'p.a.' is available, still γ-cyclodextrin is an expensive alternative to soluble starch.

4.4 Sample preparation was simplified compared to pharmacopoeia methods

Within PAT, measurements need to be simple and quick. Thus, the capability of ultrasonic spectroscopy to determine enzyme activity in real-time provides a basis for PAT. In contrast, the determination of amylase or protease activities according to pharmacopoeias in one sample requires approx. 40 min since long incubation times and several numbers of dilution steps are involved. The necessary times are reduced by determining several samples simultaneously as dead times (e.g. incubation) can be utilized. However, PAT samples need to be analyzed successively, so that interlacing of several steps is not possible. For lipase and trypsin activity measurements according to pharmacopoeia (titration methods), approx. 7 min per sample are needed. Comparable to ultrasonic methods, the required assay times do not change with increasing number of samples. In order to minimize analysis times for all enzyme classes, sample preparation should be quick and automatable.

According to pharmacopoeias, the samples have to be grinded with sea sand and a few droplets of the sample solvent before the total volume of sample solvent is filled up in order to ensure complete moistening. This preparation step was omitted for ultrasonic application to avoid resonator damages and disturbances of the ultrasonic signal caused by the sand particles. Due to up to fourty-fold smaller sample volumes used for ultrasonic methods as compared to those used in pharmacopoeia methods, incomplete moistening was rarely observed. If dissolution is not complete after 10 min, the extraction time can be extended based on feasibility tests

(3.2.4.4, **Appendix A XV**). As the grinding step is omitted in ultrasonic methods, an automatic sample dilution within PAT is easily conceivable.

Based on pharmacopoeias, extraction times of 15 min are necessary to prepare the solution of pancreas powder-containing product. Therefore, 10 to 15 min were also applied to ultrasonic methods (3.2.4.4). Analysis times were still reduced by using ultrasonic methods since only one sample solvent and one substrate solution is needed for the real-time-monitoring using ultrasonic spectroscopy, in contrast to Ph.Eur. methods which need a multiplicity of further reagents and steps (Table 37). However, substrate solutions must be degassed for HR-US measurements to avoid air-bubbles which can disturb ultrasonic measurements (4.12). For URT measurements degassing was not necessary to obtain realiable measurement results.

In order to obtain an adequate ultrasonic signal, the samples were diluted less than those analyzed with the pharmacopoeia methods. As BAEE is readily soluble, the injection of undiluted in-process samples was possible for the determination of trypsin using a build-in system (URT) in combination with the cell injection method (Figure 31). Besides injecting the enzyme solution into the cell filled with substrate solution, cell filling can also be performed by using a pre-mix of substrate and enzyme solution such as used for FTS measurements. Since the substrate volume can indefinetly be extended, using the pre-mix method theoretically allows the injection of any sample concentration and therefore the injection of undiluted samples for all enzyme classes. However, the injection of undiluted samples should be considered critical. Compared to the ultrasonic determination of lipase, protease or amylase activities, the determination of trypsin activity showed more disturbances, particularly concerning the analysis of proteolysis II material (Figure 36). This fact is supposably caused by the higher sample concentration and the lower content of buffer in the enzyme solution leading to an intense influence of the sample matrix. Using the example of amylase, it was shown clearly to what extent the sample solvent (Phosphate buffer solution (URT/ HR-US) or Phosphate buffer solution 0.2 M (URT/ HR-US)) influences the ultrasonic measurement results (Table 18).

4.5 Selectivity of ultrasonic methods is suitable for determination of enzyme activity of a single enzyme class in pancreas powder

Pancreas powder is a complex mixture of several enzymes which are categorized in the three main enzyme classes lipases, amylases and proteases. During ultrasonic measurements, simultaneous reactions of the three enzyme classes cannot be distinguished. Thus, the use of specific substrates has to ensure that secondary reactions do not proceed. In chapter 1.5 the use of the terms specificity and selectivity is explained. Tests of specificity/ selectivity were indispensable to evaluate the feasibility of measuring changes of ultrasonic velocity for the determination of lipase, amylase or protease activities in pancreas powder. Initial specificity

tests were done with systems to which

- enzyme was not added,
- substrate was not added and
- substrate and enzyme were added (positive control).

The first case was tested to ensure that no effects of temperature, volume addition, substances contained in the sample solvent as well as the substrate solution itself disturb the ultrasonic velocity measurements. The second case assured that no secondary reaction, caused e.g. by another pancreatic enzyme (sample matrix), is simultaneously detected. For all enzymes, ultrasonic velocity significantly changed only in the complete systems, but not in those that lacked substrate or enzyme (Figure 18, **Appendix A V**).

The signal strengths (change of ultrasonic velocity over time) of the negative controls were mostly far below 1 % of those of the positive controls. Besides the trypsin method, the lipase method showed the highest selectivity, supposably due to the five-fold higher signal as compared to the other enzyme determinations. The high selectivity of the trypsin method is attributed to the stable and clear substrate solution of BAEE. For total protease, approx. 5 % of the ultrasonic signal observed for the positive control was monitored by adding the activation mixture without working standard to the casein solution. This can be explained by the simultaneous monitoring of enterokinase activity. Subtraction of a blank was shown to be suitable to eliminate the superposed enterokinase signals (Table 25). Since the Ph.Eur. method, which also detects enterokinase activity, does not apply its subtraction, analysis and subtraction of blanks were not included in the final ultrasonic method to determine total protease activity. With the amylase method, about 4 % of the ultrasonic signal observed for the positive control was monitored for the system that lacked enzyme. The uncertain stability of the highly concentrated starch solution might be one reason for this fact. Within FTS measurements, the 6 % (w/v) starch solution was obviously found to be instable (strong turbidity and flocculation approx. two hours after preparation) since higher substrate volumes as compared to the build-in system methods were applied. Thus, the concentration was reduced to 3 % (w/v) for later measurements.

To sum up, the initial tests demonstrated high selectivity of the ultrasonic methods for the determination of lipase, free protease and trypsin in pancreas powder, as proven for working standard (API). Slightly lower selectivity was observed for the other ultrasonic methods (amylase, total protease).

4.6 Reliable determination of pancreatic enzyme activities in drug product and active pharmaceutical ingredient is demonstrated by comparison with pharmacopoeia methods

Feasibility tests were performed to find influencing factors and sources of error during ultrasonic measurements. Based on these tests, methods for the determination of amylase, protease and lipase activity were defined. In order to evaluate the methods, determinations of pancreatic enzyme activities in API and drug product were performed using ultrasonic and pharmacopoeia methods (chapter 3.4). Working standard was used for calibration. Furthermore, these tests served to check the capability of the ultrasonic methods for release testing and stability control.

In order to compare the results obtained with ultrasonic and pharmacopoeia methods, deviations were calculated as percentage of URT results compared to pharmacopoeia results. Preponderately, the percentage deviations were between 0 and 4 % (Table 28 - 29, Table 33 - 36). For product VII, a high percentage deviation of 8.6 % was observed concerning amylase activity (Table 33), that can be attributed to an instable 6 % starch solution (sample was the last one determined on the analysis day). Although stability tests pointed at stability over 24 hours (Table 24), in this trial the starch solution had already shown slight turbidity 8 h after preparation. Therefore, a 3 % starch solution is recommended and the starch preparation needs to be checked for turbidity before each measurement. Compared to the determination of amylase and lipase (Table 28 - 29, Table 33 + 34), higher percentage deviations were observed for the determination of free or total protease activity (Table 28 + 29, Table 35 + 36). A possible explanation might be the fact that the repeatability of the pharmacopoeia assays is relatively high, caused by the complicated sample preparation including several dilution steps and filtration.

For all enzyme classes, higher activities were observed in most cases with the pharmacopoeia methods. This fact can be attributed to the time point of analysis, as determination according to pharmacopoeias had mostly been performed a long time before ultrasonic measurements. As an example, there was a delay of approx. one year for the determination of total protease activity in Product III-VII (Table 36).
In summary, a comparison between ultrasonic and pharmacopoeia results showed that both methods were comparable. The use of ultrasonic spectroscopy to determine enzyme activity in drug product is conceivable for PAT, but also for release testing or stability tests.

4.7 Is the selectivity of the ultrasonic methods sufficient for the determination of pancreatic enzyme activities in liquid in-process samples?

The high selectivity of ultrasonic methods for the determination of amylase, lipase, protease and trypsin activity in API has been demonstrated (4.3). Selectivity refers to the extent to which a method can determine particular analytes in a complex mixture without interference

from other components (CITAC/ Eurachem, 2002). In order to evaluate possible interferences the composition of liquid in-process samples derived from the beginning of the API process needed to be considered. In order to obtain pancreas powder, proteolytic cell disruption releases pancreatic enzymes from the glands into an aqueous, buffered medium (1.2). Sodium, calcium and isopropanol are expected to be found in process samples from the beginning of the API process, as these components are added to the proteolysis make-up. Furthermore, fats and proteins introduced via glands have to be considered. Thus, the initial specificity tests (**Appendix A V**) had to be extended concerning these matrix components. Their influence was tested by determining working standard solutions which contained diverse amounts of the substances expected (Table 14 -18).

The ultrasonic methods are robust towards sodium or calcium chloride in the sample matrix, as measurements of ultrasonic velocity change over time were independent from varied content of both salts in the sample solvent (3.2.1). However, when high concentrations of calcium are present during ultrasonic determination of lipase activity two disadvantages arise. An increased ionic strength often leads to reduced emulsion stabilities (reduced zeta potential). While an adequate stability of the emulsion was obtained using 60 mM $CaCl_2$ (2.1.3.2), the precipitation of calcium soaps led to intractable deposits in the measurement cells reducing the amplitude of the resonance peaks. Removal of the deposits was time-consuming but possible by gentle rubbing with cotton buds and rinsing with surfactant and water. Cleaning of the FTS was more problematic as the mechanical step was not applicable. Nevertheless, 60 mM calcium had to be added to the system to reduce analysis times. The ultrasonic methods are also very robust towards isopropanol and proteins (model casein) in the sample solvent, except for the trypsin method in the presence of isopropanol. At this, the tests showed a slight increase of initial slopes accompanied by high CVs of up to 17 % at isopropanol concentrations above 16 % (w/w). This fact can be one reason for the unreliable results obtained for some proteolysis samples (Proteolysis II), discussed elsewhere.

In contrast, the presence of fats (model olive oil) led to several disturbances of ultrasonic measurement requiring several additional tests (Table 18). No negative influence of fat in the sample matrix was observed for lipase. This means, additional, optionally non-emulsified, olive oil had no influence on the emulsion stability (Table 18).
Concerning the determination of free protease, a slight increase of ultrasonic velocity change over time was obtained, which was significant for concentrations higher than 5 % (v/v) olive oil in the solvent (Table 18). Thus, the reaction of lipases was simultaneously detected, as the olive oil serves as substrate for lipases. Casein used as protease substrate supposably acted as emulsifier and provided the olive oil in emulsified form to the lipase. However, the effect was small and critical for high fat concentrations that are only expected when injecting a sample from an early process step straightly without previous dilution in Borate buffer (URT/ HR-US).

As opposed to the free protease method, the olive oil did not disturb the ultrasonic determination of total protease activity (Table 18). This can be explained as follows:

- The dilution of the sample with enterokinase solution minimized the total fat content in the sample cell.
- Lipolysis had already proceeded during activation time (35 °C, 15 min) previously to ultrasonic measurement.

The emulsification of olive oil in Calcium chloride solution (URT/ HR-US) was inadequate, as indicated by slight turbidity, creaming effects and oil agglutinations deposited at the mixer wall. In order to ensure better emulsification of the olive oil for better availability of the substrate for lipolysis, the experiment was repeated with addition of Triton X 100. Again, no influence of fat on the protease activity was found. Thus, the ultrasonic method to determine total protease activity is robust towards fat.

For trypsin, a high increase of ultrasonic veloctiy change over time was observed with increasing fat content due to simultaneous detection of lipase activity (Figure 20). This effect was eliminated using 1 mM hydrochloric acid as enzyme solvent without Triton X 100 (Table 18). However, with this solvent the enzyme solution merely showed slight turbidity, creaming effects were observed and there were oil agglutinations deposited at the mixer wall indicating incomplete emulsification of the olive oil in the sample solvent. As process samples contain several emulsifying agents such as proteins, mono- or diglycerides, fats could be made available for lipolysis during trypsin determination. Consequently, an enzyme solvent which contains an emulsifier such as Triton X 100, is a more appropriate model to simulate the in-process samples. Other enzyme solvents were tested as alternatives for the determination of trypsin activity. While calcium chloride showed similar effects as hydrochloric acid in the presence and absence of Triton X 100, the use of borate buffer and phosphate buffer without adding an emulsifier resulted in an increase of initial slopes in the presence of olive oil (Figure 21). Concluded, ultrasonic velocity change increased when olive oil was available in emulsified form. In the next step, blanks were obtained without BAEE. The blanks showed the same behavior as complete samples, i.e. the change of ultrasonic velocity increased with ascending olive oil content. Indeed, subtracting the blanks minimized the fat influence but did not eliminate it (Table 18). Therefore, monitoring the blank was not sufficient to solve the problem. The clear effect of fat on the ultrasonic determination of trypsin activity can be another reason for the unreliable results obtained for some proteolysis samples (Proteolysis II) (Figure 36), discussed elsewhere.

In case of amylase, an opposed behavior towards olive oil was observed (Figure 19, Table 18). The initial slopes decreased with increasing olive oil content in the enzyme solvent. Consideration of blanks was not suitable since they showed an increase of intial slopes with

increasing olive oil content. Using 0.2 M phosphate buffer, which was also adopted to eliminate the protein effect, initial slopes remained constant when the olive oil content in the sample solvent was varied. As the emulsification of olive oil in Phosphate buffer 0.2 M (URT/ HR-US) could not be accomplished, the independency of the ultrasonic measurements on the fat content was confirmed after adding emulsifier (Triton X 100) to the sample matrix. Thus, the amylase method using Phoshpate buffer 0.2 M (URT/ HR-US) is robust towards fat content in the sample matrix. Based on the results, straight application of in-process samples without further dilution is regarded as critical for amylase determination due to the dependency of emulsification and ultrasonic velocity change on the sample solvent.
In summary, the selectivity of the methods seems to be suitable for the determination of amylase, protease and lipase activities in in-process material so that no effects of fats are expected. But ultrasonic determination of trypsin activity is supposably not reliable yet in the presence of fat.

4.8 Is determination of pancreatic enzyme activity sufficiently reliable for in-process samples?

Within feasibility tests, the influence of different components which are present in in-process samples of early API process steps was tested by adding these components to working standard solutions. While the fat influence was eliminated for the determination of amylase activity in the model sample by using a higher concentrated buffer as sample solvent, the determination of trypsin was not reliable in the presence of fat and isopropanol. In-process samples differ from model samples in texture and content of fibers. Thus, the developed methods were used for determination of pancreatic enzyme activity in proteolysis and proteolysis-like material using working standard for calibration. In some cases, reference measurements with pharmacopoeia methods were obtained. While lipase determination reliably worked (Figure 35, Table 27), determinations of the other pancreatic enzymes differed according to sample material and used method. All enzyme activities showed a plausible course of enzyme activity during proteolysis but no direct dependency on the solid content.

Using Phosphate buffer (URT/HR-US) as enzyme solvent for the ultrasonic determination of amylase activity, lower relative amylase activities of approx. 7,000 Ph.Eur.-u/ g were measured in the proteolysis-like material (Figure 26) as compared to proteolysis samples (Proteolysis I) with an average relative amylase activity of approx. 9,000 Ph.Eur.-u/ g (Figure 29). Due to the optimized conditions for proteases (trypsin) in the proteolysis-like material, lower amylase activities in the proteolysis-like material are plausible. Comparing the first proteolysis experiment (Proteolysis I) with the second proteolysis experiment (Proteolysis II), one-tenth higher amylase activities (approx. 10,000 FIP/Ph.Eur.-u/g) were observed using the more concentrated buffer (Phosphate buffer 0.2 M (URT/ HR-US)) as sample solvent, measuring the samples without

previous separation of fibers, using the Pre-mixing method and applying a 3-point-calibration (Figure 32). Furthermore, the CVs within one batch were reduced from averaged 7 % to 1.5 %. The variations within the three proteolysis batches were distinctly reduced in Proteolysis II. Robustness measurements of the feasibility studies concerning fat content proved a loss of 40 % amylase activity at high olive oil contents. Thus, the difference of 10 % observed between the first and second proteolysis experiment may be caused by the fat content of the in-process samples. Additionally, the variability of the porcine glands introduced may lead to differences in amylase activity during proteolysis. Reference measurements of selected Proteolysis II samples using the Ph.Eur. method showed approx. 20 % reduced activities compared to the ultrasonic method results (Table 27). The sample matrix might disturb the Ph.Eur. method. Another reason might be the time gap between both measurements, as the measurements according to Ph.Eur. were carried out one month later with frozen samples (defrosted before measurement).

As expected, free protease activity increased over time during proteolysis due to activation processes (Figure 33). Higher free protease activities of approx. 600 Ph.Eur.-u/ g were determined in the proteolysis-like material (Figure 27) as compared to proteolysis samples (Proteolyse I) with free protease activities of approx. 350 Ph.Eur.-u/ g (Figure 30). Higher activities in the proteolysis-like material are plausible, because activated trypsin is enhanced due to optimized conditions for proteases. Comparing the first proteolysis experiment (Proteolysis I) with the second proteolysis experiment (Proteolysis II), comparable free protease activities were obtained (Figure 30, Figure 33). Implementation of the Pre-mixing method and applying a 3-point-calibration led to improved CVs, reduced from averaged 4.5 % to 2.5 %. Using the Ph.Eur. method, unreliable results were obtained within reference measurements of selected Proteolysis II samples. Free protease activity decreased in the course of proteolysis (Table 27) indicating the limit of the Ph.Eur. method. Another reason might be the time gap between both measurements, since the measurements according to Ph.Eur. were performed one month later with frozen samples (defrosted before measurement). The decrease of total protease activity over time during proteolysis results from auto-digestion of the activated proteases (Figure 34). Reference values obtained with Ph.Eur. method showed same tendencies (Table 27). However, percentage deviations of ultrasonic method in relation to the pharmacopoeia method of up to 20 % were observed, supposably caused by the time gap between ultrasonic and pharmacopoeia measurements.

Trypsin activity increased over time during proteolysis due to activation (Figure 36). Higher trypsin activities up to 500 Ph.Eur.-u/ g were determined in the proteolysis-like material (Figure 28), since in proteolysis samples (Proteolyse II) free trypsin activities of 100 Ph.Eur.-u/ g were observed (Figure 36). Higher activities in the proteolysis-like material are plausible as activated trypsin is enhanced due to optimized conditions for proteases. A comparison of ultrasonic measurement results with those obtained with Ph.Eur. method showed differences

for proteolysis-like (Figure 28) and proteolysis II material (Figure 36). When proteolysis-like material was analyzed, ultrasonic measurements gave similar results as the Ph.Eur. method. Merely lower activities were found using the ultrasonic method. Whereas the Ph.Eur. method was performed with freshly sampled material, frozen material was used for ultrasonic measurements carried out some weeks later. This fact might be the reason that the first sample of each batch showed particularly high percentage deviations of ultrasonic method in relation to the pharmacopoeia method. This is supposably caused by the fast change of trypsin activity over time due to activation processes. Other percentage deviations ranged within 3 to 5 %. In contrast, high percentage deviations of about 30 % were obtained for free trypsin activity in proteolysis II material. Whereas the trypsin activities determined by Ph.Eur. method had a similar time course, high variations within the three batches and outliers (3 - 4 hours) were observed for ultrasonic measurements (Figure 36). This was not expected as proteolysis II material was dissolved in 1 mM HCl to reduce fat influences by lacking Triton X 100 from the sample solvent. Thus, an optimization of the determination of trypsin activity would have been expected from proteolysis-like to proteolysis II material. Determining trypsin activity of the working standard, ultrasonic and pharamcopoeia methods led to different activities of the working standard (URT: 1,296 u/ g, Ph.Eur.: 1,387 u/ g). In order to check this fact as reason for the high percentage deviations of ultrasonic method in relation to Ph.Eur. method, the trypsin activity based on Ph.Eur. was also used to calculate the relative trypsin activities of proteolysis II material. Only slightly improved percentage deviations were obtained.
The ultrasonic method to determine trypsin activity differs from the other ultrasonic methods in sample dilution. Samples are only diluted approx. twofold, which leads to a higher influence of the sample matrix compared to other ultrasonic methods. Therefore, more emulsified fat and more isopropanol are present in the measurement cell influencing the ultrasonic measurement result. Disturbances were more intense for the proteolysis II material as compared to the proteolysis-like material. Using the Pre-mixing method was one difference between both determinations, but since analysis of working standard solutions was not conspicuous this difference does not seem to be the reason for the different behavior. Furthermore, Triton X 100 was not introduced for proteolysis II material. In feasibility test, the lack of Triton X 100 led to minimized fat influences. Thus, the reasons caused the disturbances in measurement seem to be others: First, fibers were included, which supposably led to higher variations. For the other ultrasonic methods the fibers were uncritical as samples were diluted strongly. Secondly, the different sample composition such as higher fat and isopropanol content in proteolysis material compared to proteolysis-like material, might have caused more disturbances.

In conclusion, for lipase, amylase and protease plausible results were obtained in proteolysis material. Nevertheless, the tests demonstrated the complex interplay between different matrix components. In part, this led to unreliable results with both the ultrasonic (trypsin) and the pharmacopoeia methods (amylase, protease). For the determination of trypsin, the disputable

measurement results confirmed the feasibility tests. It was clearly shown that further research is needed concerning the ultrasonic trypsin method.

4.9 The pre-mixing method leads to improved precisions

Precision is one important parameter to judge and ensure quality and reliability of analytical results. ICH (1994) distinguishes repeatability, intermediate precision and reproducibility (1.5). Repeatability and intermediate precision were determined for ultrasonic methods using working standard (API). Repeatability was found between 1 and 2 % for both build-in system devices (URT, HR-US) and all ultrasonic methods (Table 19). As a result, the repeatabilities were highly comparable with those of the pharmacopoeia methods. Except for lipase determination, best repeatability of less than 1 % was observed using the Flow-through System HR-US FTS (Table 19). As ultrasonic velocity depends on temperature, reasons for the improved precisions are supposably the permanently closed measurement cells and the pre-conditioning of the fluids before reaching the cells (Figure 9). Concerning determination of amylase activtiy, the reduced starch concentration might have also influenced the result. A 6 % (w/v) starch solution was not suitable for FTS measurements since instabilities occured. These instabilities were supposably caused by the higher volume in the substrate reservoir bottle as compared to the volumes needed for HR-US measurements in the build-in system. Furthermore, the backflow into the bottle, i.e. alternately tempering to 37 °C and cooling down at room temperature, could have depressed the stability of the starch solution. While testing repeatability of the lipase method, a continuous decrease of activity over time was observed from the analysis of the first to the analysis of the sixth working standard solution accompanied by deposits of calcium soaps in the system. This effect might be the reason for the fact that the repeatability was not improved using the FTS compared to the use of the build-in system for lipase determination (Table 19).

As a result from working with the FTS, the Pre-mixing method was also applied to the URT and HR-US spectrometer (build-in systems) (Table 19). The repeatability improved, supposably caused by the higher volume of enzyme solution which is pipetted with less variability as smaller volumes. Furthermore, mixing of substrate and enzyme solution in the sample cell often induces the generation of air bubbles which can initiate disturbances of ultrasonic measurements.

Concerning total protease activity, Born *et al.* (2009) observed repeatabilities of 5 % and higher using URT. Similar to the pharmacopoeia method (2.2.2.3), Born *et al.* performed several sample preparation steps (e.g. incubation of casein with enzyme, stopping the reaction with TCA and centrifugation). Afterwards, ultrasonic veloctiy in the supernatant was measured as difference to a blank (1.4.4). Since ultrasonic velocity in the samples was measured once after stopping the reaction, slight variations in e.g. the incubation time could have influenced the measurement result. In contrast, for the present thesis ultrasonic spectroscopy was used to monitor protease

activity in real-time. This led to improved precisions.

For intermediate precision measurements, no calibration with working standard was done but absolute ultrasonic velocity changes were used to calculate intermediate precisions. They were improved using the pre-mixing method and range between 2 and 3 %. Having reached adequate intermediate precisions optionally makes calibration on each analysis day unnecessary.

4.10 Tempering systems are sufficient, but influence analysis time

Linearity is another important parameter to judge and ensure quality and reliability of analytical results. The linearity of an analytical procedure is its ability to obtain results which are directly proportional to the concentration or potency of the analyte (ICH Q2 (R1), 1994). Testing linearity of the ultrasonic methods, linearity was found in a broad range for all methods. The calibration lines were no lines through the origin (Figure 22). For the ultrasonic determination of API, one- and three-point calibration were tested (Table 28 + 29). In most cases, the one-point calibration led to lower results as compared to the pharmacopoeia methods, whereas no tendency was observed for the results obtained using the three-point calibration. Furthermore, results calculated using the three-point calibration showed smaller percentage deviations of ultrasonic method in relation to pharmacopoeia method as compared to the results calculated after one-point calibration. Consequently, the performance of one-point calibration led to less accurate results than using three-point calibration.
In order to obtain accurate results by one-point calibration, equal changes of ultrasonic velocity over time for the analysis of working standard (calibration) and sample are required. In the case of non-homogeneous samples, this demand is difficult to meet. In contrast, three-point calibration allows a broader range of enzyme solution concentration (within the linear range) and leads to more reliable results when the change of ultrasonic velocity (m/s^2) of the sample over time is not identical to that of the reference standard.

4.11 Methods are robust towards small temperature variations

Given its relation to density, ultrasonic velocity strongly depends on temperature (1.4.3). Coupland (2004) dealed with the inadequate temperature control as a common problem of low-intensity ultrasonic devices, which can be excluded by holding a reference cell in close thermal proximity to the sample cell. Thus, in order to ensure no influence of small temperature changes the enzyme activity measurements were always performed by using one cell filled only with substrate and one cell filled with the substrate-enzyme-mix. When enzyme solution is added to the sample cell, temperature in the sample cell decreases faster than in the reference cell as the sample cell has been opened and ice-cold enzyme solution has been injected into the cell. After closing the cells, the difference between both cells is adjusted within a defined time period, which is characterized by a non-linear course of ultrasonic velocity change. Afterwards,

the temperature in both cells increases to the set temperature while a linear course of ultrasonic velocity change is observed. In this latter stage, temperatures below 36.8 °C have not been observed. Small temperature differences tested in the range from 36.8 °C to 37.2 °C showed no influence on the enzyme activities (2.2.4.1, **Appendix A XI**) allowing measurements to start immediately after temperature adjustment of the sample cell to that of the reference cell. The time which was necessary for adjustment was determined as 40 sec (URT), 200 sec (HR-US) and 100 sec (FTS), respectively, as proven within specificity tests when no enzyme was present.

Short analysis times are essential for the use of analytical methods within PAT. Therefore, the pharmacopoeia methods are not applicable as PAT method (4.4). In contrast to pharmacopoeias, ultrasonic methods are time-saving as they monitor the enzyme reaction in real-time and need only few reagents.
Due to another tempering system and smaller cell volumes (1.4.3), the URT device allowed faster measurements than the HR-US spectrometer (Table 38). Analysis times of the build-in system were reduced with HR-US FTS, supposably due to pre-conditioning of the substrate-enzyme-mixture in the instrument (1.4.3). Tempering the cleaning, substrate or enzyme solutions prior to HR-US measurements showed no influence on the time consumption. The pre-mixing method led to small improvement of analysis times as the beginning of the evaluation area was shifted from 250 to 100 sec after enzyme addition. Potentially, analysis times can be improved by reducing the evaluation area from current 200 sec to shorter times (e.g. 50 sec).

Table 38. Times needed for basic steps of URT and HR-US measurement (without negligible times for filling and cleaning).

	URT	HR-US
Instrument preparation (once a day)	5 min	2 min
Baseline check	1.3 min	*5 min
Measurement of enzyme kinetic	4 min	8 min

* Using the FTS or filling the cells of the conventional ultrasonic devices via Pre-mixing method the analysis time is reduced due to the omitted baseline check

Summing up, temperature control was adequate for both devices during determination of enzyme kinetics, extensively proved in precision and specificity tests. However, the tempering system influenced the analysis times leading to 2.5-fold longer analysis times using HR-US in comparison to URT measurements. In view of PAT, reduction of analysis times for HR-US is necessary.

4.12 A Flow-Through System pioneers complete automation

The feasibility to determine enzyme activity in different kinds of samples using the build-in systems of URT/ HR-US devices has been shown (3.4), which would allow an at-line application

of both devices for PAT purpose. In order to use the ultrasonic technique as on-line measurement as well, an FTS was tested. The use of the FTS implicated the necessity to pre-mix substrate and enzyme solution (Pre-mixing method) and to subsequently rinse the pre-mix through a tubing system into the resonator cells.

Using working standard (API), repeatability and analysis times were improved with FTS in comparison to the use of the build-in systems (Table 19). Adopting the Pre-mixing method, theoretically enables the application of any sample concentration due to an unlimited substrate volume. As an example, a highly concentrated sample was introduced for the ultrasonic determination of amylase activity, adding a reduced volume of sample to the starch solution. The repeatability was debased but adequate (below 1 %) (3.2.2). Although liquid process samples could be applied without previous sample dilution (Table 20, Figure 31), the addition of the sample solvent as buffer is adjuvant to avoid matrix effects. As an example, Phosphate buffer 0.2 M (URT/ HR-US) eliminated fat disturbances during monitoring amylase activity (Figure 19).

Handling and cleaning of the system with water and a low concentration of surfactant solution was generally easy and quick, as required for PAT use. However, calcium soaps precipitated during lipase reaction led to deposits in the cells, which were hard to remove, since mechanical cleaning was not possible. Conveniently, beginning of precipitation appears in reversion of attenuation (Figure 38). Initially, ultrasonic attenuation decreases during lipase reaction since the droplet size is reduced due to the degradation of olive oil (1.4.5). Then, precipitated calcium soaps enhance the wave scattering leading to a decrease of amplitude (and an increase of attenuation). Hence, maintaining exposure times in the FTS below 420 sec and subsequently rinsing with surfactant and water can avoid deposits of calcium soaps.
Considering the determination of enzyme activity in liquid IPC samples, the effect of fibers in the samples regarding passage through the tubes and consequential cleaning issues needed special attention. After injecting pancreas dispersed in water into the FTS without previous

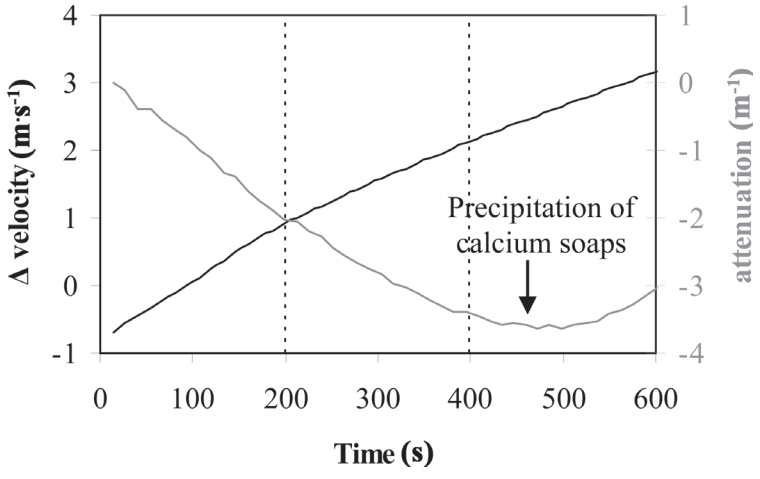

Figure 38. Lipase reaction was monitored by measuring the change of ultrasonic velocity (black) and attenuation (grey) over time. Initially, ultrasonic attenuation decreased due to droplet size reduction. After 450 sec attenuation reversed caused by the precipiation of calcium soaps.

filtration (Table 20), fibers got caught in the tubing material, especially in the tube adapters. To remove the fibers by rinsing out the system was difficult. Thus, the adapters had to be removed, cleaned and reattached to the system. It is also expected that fibers got caught inside the FTS as fibers were still found one day later although the system was rinsed for several hours. When the fibers were separated from the samples, no cleaning problems were observed. Furthermore, repeatabilities of the measurement were improved (Table 20). Thus, for the determination of highly fibered IPC samples, previous fiber separation is recommended. In laboratory scale, fiber separation was done via dissolution basket. Concerning automation, commercialized filtering systems (Figure 39) are available (e.g. by Flownamics®, Trace Analytics GmbH, Eppendorf AG), generally offered to obtain cell-free or sterile samples from bioprocesses. A replacement of the membrane with a tubular sieve may lead to an effective separation of crude fibers without losses of enzyme activities. Alternatively, a set-up avoiding adapter pieces and featuring large tube diameters is conceivable to ensure an adequate cleaning. However, enlarged diameters of the tubing material would consequently lead to an unacceptable increase of analysis time.

In addition to FTS, automation was tested in another manner. Tests using a dosing apparatus and titration equipment showed a loss of enzyme activity (tested for amylase and protease) after long holding times (more than 20 min) of the enzyme solution in the dosing system (Figure 23). This decrease can be attributed to either slow absorption to tubing or syringe material or to the long exposure time to room temperature. Since the tubing material is known as biocompatible, the latter case seems to be more likely. This effect of long duration times (more than 20 min) has to be considered for planning the use of an autosampler in the laboratory or the sampling (branch line) in the process.

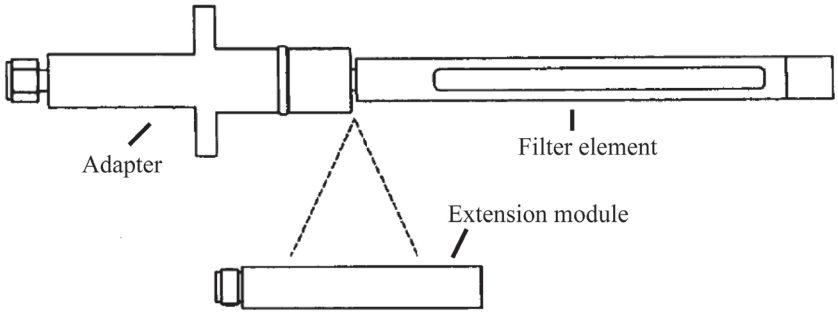

Figure 39. Set-up of sampling device with filter element part, which sticks into the medium. Special extension modules can be used to elongate it (Scheper *et al.* (1996).

In view of PAT, an adequate stability of substrate solutions is needed to ensure a continuous operation. Stability for three weeks was proven for the olive oil emulsion. However, tempering the emulsion to 37 °C continuously has a negative influence on the stability. As opposed to the olive oil emulsion, the stability of starch solution is enhanced when the solution is stored in a waterbath at 37 °C. Nevertheless, starch preparations were often stable for only one day depending supposably on the water content of the used starch. The pharmacopoeias require daily preparation of fresh starch solution. Although the same is specified for casein solution,

casein solutions are sometimes stable for several days (proven for 96 hours). Incipient turbidity and flocculation of the starch or casein solutions indicate the expiration of use for ultrasonic methods. Stability of BAEE solution was tested over 48 hours and longer physical stability is expected. Chemical instability during long operation times is estimated to be uncritical as a broad range of substrate concentrations was identified with no effect on the change of ultrasonic velocity (Table 23) wherat the operating substrate concentrations are in the middle or at the end of the tested ranges.

Given the impossibility to determine enzyme activity in-line, the use of an auto-sampling device is also part of automation. In this work, autosampling has not been considered in the practical section but should be addressed in future studies. Auto-sampling is needed for sampling, dilution of the samples, and pre-mixing with substrate solution. Hansen (2004) gave a helpful overview about sampling technologies. Flow Injection Analysis (FIA), developed by Ruzicka & Hansen (1981), is a sampling technique based on discrete sampling by injection into a continuously flowing carrier stream, which may be merged downstream with reagents, transporting the sample to the analyzer unit. Separation of each sample from the subsequent sample is accomplished by the carrier reagent (Ruzicka & Hansen, 1981), bordering from the gas-segmented flow analysis according to Skeggs (1957) which uses air bubbles for separation. In the literature, enzyme assays are often described in combination with FIA in which the enzyme reaction is performed prior to injecting the reaction products into the flow system. Nicolas *et al.* (1990) described a method to determine proteolytic enzymes including the mixing with substrate, enzyme reaction and UV spectroscopic detection within the FIA (Figure 40).

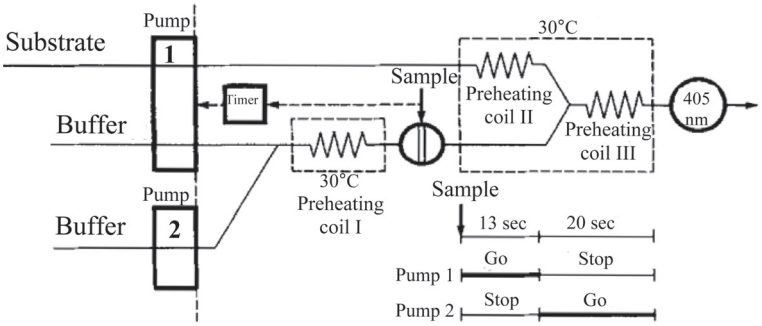

Figure 40. FIA-manifold for proteolytic enzymes determination and schematic representation of the timer program. The sample was injected into the buffer stream which was then merged with the substrate stream before reaching the measurement cell. A second pump was used to clean with pure buffer at a high flow rate (according to Nicolas *et al.*, 1991).

In the 1990s, development of the FIA concept has led to new generations: SIA (Sequential Injection Analysis) and LOV (lab-on-valve), which use programmable, bi-directional discontinuous flow instead of continuous flow via a multi position selector valve (Figure 41). Due to miniaturization of the manifold, sample and reagent consumption are smaller than using traditional FIA, but the analysis time may be longer caused by the syringe pump (Hansen, 2004). Additionally,

LOV features an integrated microconduit, designed to perform all necessary unit operations required for a given assay such as mixing points for the analyte and reagents (Hansen, 2004). Cos *et al.* (2000) developed an on-line monitoring of lipolytic activity using SIA. A stop-flow technique was used to increase the conversion of the enzymatic reaction. Therefore the authors stopped the flux for 10 min in the heating coil (reactor) before passing the detector.

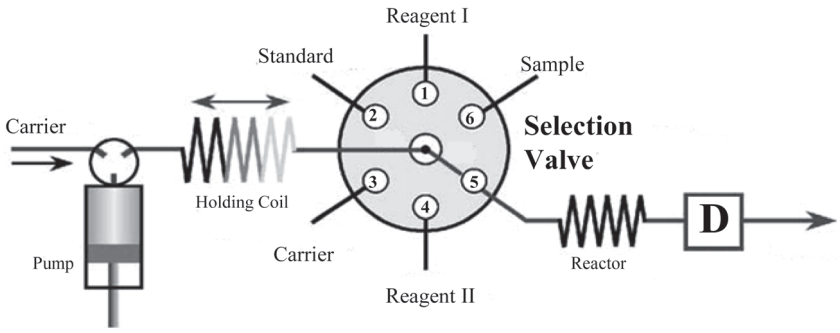

Figure 41. Typical SIA system based on selection valve and bidirectional syringe pump [D = detector]. For explanation see text (Hansen, 2004).

The stopped-flow mode is also used to monitor the enzymatic reaction in real-time, whereas the reaction mixture is held within the flow-through cell for monitoring (according to FIAlab®). Several commercial instruments for SIA and LOV are available on the market, but mostly in combination with UV/Vis spectroscopy or fluorescence (e.g. FIAlab®). No literature was about SIA and LOV for ultrasonic measurements.

In summary, FTS measurements have led to a better repeatability of ultrasonic measurements as compared to the build-in systems. Using the titration equipment, instabilities of pancreas powder-containing samples were identified leading to amylase and protease activity losses after approx. 20 min. Concerning PAT, this is not relevant as PAT requires data to be obtained virtually in "real-time". Long time intervalls between sampling and analysis are not suitable for PAT as they make a prompt intervention impossible. Adopting FIA or SIA/ LOV for autosampling might allow the complete automation of the system. Due to short holding times of the sample in the FIA system, enzyme activity losses can be eliminated. Kroner (1988) refers to this advantage, particularly concerning enzyme analysis at high temperatures.

4.13 Ultrasonic measurements have advantages and disadvantages in view of PAT

The applicability of turbid sample preparations, quick analysis times and the possibility of automation are advantages of ultrasonic spectroscopy that have frequently been stressed in the literature and motivated to explore ultrasonic spectroscopy for the determination of pancreatic enzyme activities for quality control and PAT. As a result, ultrasonic methods proved to be applicable to pancreas und pancreatic powder-containing samples with advantages and disadvantages as compared to pharmacopoeia methods (Table 39).

Table 39. Advantages and disadvantages of ultrasonic spectroscopy to determine pancreatic enzyme activities in different kinds of samples.

Advantages	Disadvantages
▪ Non-destructive technique	▪ Calibration with reference standard
▪ Analysis of turbid samples is possible	▪ Unspecific signal
▪ Simple sample preparation	▪ Stable substrate formulation is required
▪ Special structures are not required	▪ Secondary reactions influence result
▪ Analysis in real-time (→ Analysis time)	▪ Dependency on temperature (→ Analysis time)
▪ Automatable	▪ Influence of air bubbles

In the present thesis, it was shown that ultrasonic spectroscopy allows the use of turbid substrate preparations based on the pharmacopoeias, such as starch solution, casein solution or olive oil emulsion to determine amylase, protease and lipase activities in API and drug product. The great challenge was to determine the activity of a single enzyme class in the presence of both other enzyme classes and in the presence of disturbing substances in the sample matrix of liquid process samples which are e.g. substrates for one of the other enzyme classes. Only in the context of liquid proteolysis samples matrix effects were observed. The trypsin method proved not to be reliable with respect to proteolysis samples supposably caused by high concentrations of fat and isopropanol (4.5 - 4.8). Furthermore, instabilities of the substrate preparation, especially the olive oil emulsion (4.3), which were not caused by the enzyme reaction itself had to be eliminated to ensure a reliable measurement of enzyme activity. Instabilities of the starch solution could not be overcome in all instances and often led to disturbances during ultrasonic determination of amylase activity. Coupland (2004) described the simultaneous change of multiple sample variables as a practical problem concerning the use of low-intensity ultrasound.

The sensitivity of ultrasonic methods to temperature changes (Coupland, 2004) was addressed by the use of instruments that allow a reference calibrant cell to be in close proximity to the sample cell in a temperature-controlled environment. However, tempering delays led to different analysis times using URT and HR-US.

According to Coupland (2004), disturbances by air bubbles in the sample occur during ultrasonic measurements. Large air bubbles act as reflector and are imaged ultrasonically, while smaller air bubbles attenuate the sound leading to impossible propagation or prevented resolution (Povey, 1997). In order to avoid this, substrate preparations were degassed under vacuum (HR-US), however, injected samples were not degassed. Mixing was always performed carefully to avoid generation of air bubbles. Working in this way, no negative influences of air bubbles on the measurement were observed.

Another problem pointed out by Coupland (2004) is sensor erosion, especially caused by high temperatures and corrosive solvents or fouling of the transducer surface. Indeed, deposits of calcium soaps on the transducers reduced the amplitudes by 50 % in the perfomed measurements. Due to the non-applicability of corrosive solvents and the poorly visible and fixed sample cells,

cleaning was complicated. For FTS, cleaning was additionally hampered as mechanical cleaning was not possible.

The determination of absolute values of enzyme activity by ultrasonic methods is not conceivable as the ultrasonic signal during enzyme reaction is a complex interplay between substrate degradation, product generation and matrix effects (4.1). Therefore, calibration with reference standard was done and enzyme activities were determined as relative enzyme activity in relation to reference standard. Due to the complexity of the ultrasonic signal, disturbances cannot be explained easily.

Unspecific signals (instabilities of substrate preparation, secondary enzyme reactions), temperature influences and air bubbles are also problematic when other techniques such as turbidimetry or NIR are considered (1.3.2). In summary, enzyme activity measurements by ultrasonic spectroscopy is feasible for quality control of API and drug product. In addition, its use in PAT seems to be possible for amylase, lipase and protease in pancreas and pancreas powder-containing in-process samples, although more research is needed to further develop it as PAT method for the determination of trypsin in liquid proteolysis samples. Analysis times of HR-US and cleaning should be optimized.

5. SUMMARY

Ultrasonic Spectroscopy is a fast and automatable technique that uses acoustic waves of low power and high frequency propagating through a sample. Due to changes of density and compressibility of the substrate-enzyme-mixture, an enzyme reaction can be observed by monitoring the change of ultrasonic velocity over time. Enzyme activities can be determined relative to a reference standard. In order to avoid influences of temperature, reference measurements are carried out using one sample cell filled with substrate and enzyme solution and one substrate-filled reference cell. In contrast to most pharmacopoeia methods, Ultrasonic Spectroscopy does in principle not require any sample preparation as turbid and undiluted samples can be measured. The goal of the present thesis was to test the technique as in-process control in view of PAT and as an alternative method for the determination of enzyme activity in porcine pancreas powder and porcine pancreas-powder containing product.

Ultrasonic Resonator Technology (URT, TF Instruments GmbH) and High Resolution Ultrasonic Spectroscopy (HR-US, Sonas Technologies Ltd.) were used to develop ultrasonic methods to determine amylase, lipase, protease (free and total) and trypsin activities in pancreas powder and pancreas and pancreas powder-containing material, in each case in the presence of the other pancreatic enzymes.

Basic feasibility tests were carried out using a pancreas powder working standard. The temperature was defined to 37°C for all enzymes. Although adjuvants and concentrations needed to be adjusted to the requirements of ultrasonic spectroscopy, assay conditions and substrates were defined that they closely resembled those of Ph.Eur. and USP:

- Concerning the determination of protease activity, the casein preparation (1.25 % (w/v), aqueous) was adopted without modification leading to a repeatability of 1.5 % (URT: 1.71 %, HR-US: 1.25 %) for the determination of free protease activity and 1.5 % (URT: 1.45 %, HR-US: 1.48 %) for the determination of total protease activity.
- Concerning the determination of amylase activity, a substrate concentration of 6 % and the addition of buffering substances and cofactors (Phosphate buffer pH 6.8, sodium

chloride 6.7mM) to the system improved the repeatability to 2.0 % (URT: 1.86 %, HR-US: 2.28 %). Using a 3 % starch solution the repeatability was 1.5 % (URT: 1.30 %, HR-US: 1.71 %).

- Replacing the emulsifier gum acacia with Triton X 100, adding cofactor calcium, lowering the pH to 7.2 and using a high pressure homogenizer (Microfluidizer®) to process the emulsion led to a stable emulsion, applicable for ultrasonic measurements to determine lipase activity. The repeatability was 1.6 % (URT: 1.80 %, 1.43 %).
- Replacing the borate buffer with TRIS buffer as sample solvent enabled the ultrasonic determination of trypsin. The repeatability was 1.73 % (URT).

Using working standard, it was also shown that the selectivity of the developed ultrasonic methods was adequate for the determination of single enzyme activities in an enzyme mixture. The applicability of ultrasonic spectroscopy as in-process control of proteolysis was tested by adding several substances to the sample matrix. Substances contained in the sample matrix such as sodium chloride, calcium or isopropanol did not influence the enzyme activity in the developed methods. Protein (model: casein) led to an enhanced ultrasonic signal during determination of amylase activity since protease activity was monitored simultaneously. In contrast, high concentrations of fat (model: olive oil) led to reduced signals for amylase. Blanks (without starch) showed an opposing effect towards fat. Changing the buffer concentration in the sample solvent eliminated the disturbances caused by proteins and fat during amylase determination. Fat also enhanced the signal during determination of trypsin activity as lipolysis was monitored simultaneously. This was avoided by omitting emulsifying agents.

Substrate stability was adequate for lipase, protease and trypsin determination. However, instabilities of starch solutions disturbed the determination of amylase activity consistently. The increased temperature compared to the pharmacopoeia method had a positive influence on the stability of starch solution.

Time consumption for determination of two replicates using URT is 10 min and approx. 26 min using HR-US because of a higher cell volume in HR-US and a different tempering system. Automation was tested by using a Flow-through System (FTS). Since the substrate-enzyme-mixture is automatically tempered before reaching the sample cell, the use of FTS shortened the analysis time. Transferring the pre-mix method to the build-in systems of URT and HR-US also accelerated the analysis time as the baseline check was omitted and improved repeatability.

In order to compare results obtained with the ultrasonic method with those of the pharmacopoeia methods, the change of ultrasonic velocity of reference standard solution and sample solution was determined. The enzyme activity was calculated by using the activity label claim of the working

standard based on pharmacopoeias. Concerning API and drug product, the enzyme activities obtained with HR-US and URT were comparable with the results of the pharmacopoeia methods (Ph.Eur. and USP). This confirms the high selectivity of the ultrasonic methods. Concerning in-process proteolysis samples, the determination of amylase activity was optimized by using a sample solvent with higher buffer concentration. Lipase and protease (free and total) methods appeared to be applicable to in-process samples. In contrast, ultrasonic determination of trypsin activity in proteolysis material did not yield reliable results likely due to disturbances of fat and isopropanol because of higher sample concentrations as compared to other methods.

A comparison of ultrasonic spectroscopy with alternative techniques (turbidimetry and NIR/MIR) as described in the literature shows similar advantages as well as challenges for all techniques in view of IPC of proteolysis.

In conclusion, ultrasonic spectroscopy can be an alternative to determine pancreatic enzyme activity in most process samples, drug substance and drug product. In order to use URT or HR-US in the context of PAT for in-process control, additional experiments concerning automation (online), analysis times and elimination of disturbing matrix effects are recommended. Based on the results presented here, use of URT and HR-US for release testing of pancreas powder and porcine pancreas powder-containing product is possible.

6. ZUSAMMENFASSUNG

Hinter Ultraschallspektroskopie verbirgt sich eine schnelle und automatisierbare Technik, die Ultraschallwellen hoher Frequenz und geringer Intensität nutzt. Durch die Veränderung von Dichte und Kompressibilität des Substrat-Enzym-Gemisches kann eine Hydrolysereaktion über die zeitliche Veränderung der Ultraschallgeschwindigkeit verfolgt werden. Enzymaktivitäten können in Relation zu einem Referenzstandard bestimmt werden. Um Temperatureinflüsse zu vermeiden, werden Differenzmessungen zwischen einer Probezelle, die Substrat- und Enzymlösung enthält, und einer mit Substratlösung gefüllten Referenzzelle durchgeführt. Im Gegensatz zu den meisten Arzneibuchmethoden ist theoretisch für die Ultraschallspektroskopie keine Probenaufbereitung nötig, weil auch trübe unverdünnte Proben vermessen werden können. Im Rahmen dieser Arbeit wurde die Ultraschall-Technik in Hinblick auf PAT als Inprozesskontrolle und als alternative Freigabeanalytik zur Bestimmung von Enzymaktivitäten in Pankreaspulver und entsprechenden Produkten untersucht.

Mit dem Einsatz von URT (Ultrasonic Resonator Technology, TF Instruments GmbH) und HR-US (High Resolution Ultrasonic Spectroscopy, Sonas Technologies Ltd.) wurden Methoden zur ultraspektroskopischen Bestimmung von Amylase-, Lipase-, freier und gesamter Protease- und freier Trypsinaktivitäten in Pankreaspulver und pankreaspulverhaltigem Material, jeweils in Gegenwart der anderen Pankreasenzyme, entwickelt.

Mit einem Pankreatin-Arbeitsstandard wurde zunächst die grundsätzliche Anwendbarkeit untersucht. Für alle Enzyme wurde eine identische Temperatur von 37°C gewählt.
Obwohl Hilfsstoffe und Konzentrationen teilweise den Bedürfnissen der Ultraschall-Spektroskopie angepasst werden mussten, wurden die Bedingungen und die Substrate so weit wie möglich an den Ph.Eur.- und USP-Vorgaben orientiert:

- Für die Protease-Bestimmung wurde die Casein-Lösung (1,25 % (w/v), wässrig) ohne Änderungen vom Arzneibuch-Assay übernommen. Eine Wiederholbarkeit von ca. 1,5 % (URT: 1,71 %, HR-US: 1,25 %) wurde für die Methode zur Bestimmung der

freien Proteaseaktivität ermittelt. Für die Methode zur Bestimmung der Gesamtaktivität war die Wiederholbarkeit ebenfalls ca. 1,5 % (URT: 1,45 %, HR-US: 1,48 %).

- Für die Bestimmung von Amylaseaktivität verbesserte eine erhöhte Stärke-Konzentration von 6 % (w/v) und die Anreicherung mit puffernden Substanzen und Co-Faktoren (Phosphatpuffer pH 6,8, NaCl 6,7 mM) die Wiederholbarkeit auf ca. 2,0 % (URT: 1,86 %, HR US: 2,28 %). Mit einer 3 % (w/v) Stärke-Lösung wurde eine Wiederholbarkeit von ca. 1,5 % (URT: 1,30 %, HR-US: 1,71 %) ermittelt.
- Für dieBestimmung der Lipaseaktivität führte ein Emulgatoraustausch von Arabisch Gummi gegen Triton X 100, die Ergänzung von Calcium, eine Erniedrigung des pH-Wertes (pH 7,2) und die Anwendung eines Hochdruckhomogenisators (Microfluidizer®) zur Emulsionsherstellung zu einer stabilen Emulsion, die für Ultraschallmessungen applizierbar ist. Die Wiederholbarkeit war ca. 1,6 % (URT: 1,80 %, 1,43 %).
- Für die Bestimmung der freien Trypsinaktivität musste der Boratpuffer gegen TRIS-Puffer ausgetauscht werden, um ein Ultraschallsignal zu erzeugen. Die Wiederholbarkeit war 1,73 % (URT).

Mit Pankreatin-Arbeitsstandard konnte ebenfalls gezeigt werden, dass die Selektivität der entwickelten Ultraschall-Methoden für die Bestimmung von einzelnen Enzymaktivitäten im Enzymgemisch ausreichend hoch ist. Inwiefern die Ultraschallspektroskopie auch als Inprozesskontrolle der Proteolyse genutzt werden kann, wurde durch den Zusatz verschiedenster Substanzen zur Probenmatrix untersucht.
In der Matrix enthaltende Substanzen, wie Natriumchlorid, Calcium oder Isopropanol zeigten keinen Einfluss auf das Messergebnis. Proteine (Casein als Modell) führten durch die gleichzeitige Erfassung von Proteolyse bei der Amylase-Bestimmung zu einer erhöhten Veränderung der Ultraschallgeschwindigkeit. Höhere Fettkonzentrationen (Olivenöl als Modell) bewirkten dagegen für Amylase eine Erniedrigung des Signals, wobei Blindwerte (ohne Stärke) ein entgegengesetztes Verhalten gegenüber Fett zeigten. Eine Erhöhung der Phosphat-Pufferkonzentration beseitigte die durch Fett und Proteine ausgelösten Störungen bei der Amylase-Bestimmung. Wegen gleichzeitig ablaufender Lipolyse erhöhte Fett auch das gemessene Ultraschallsignal bei der Trypsinaktivitätsbestimmung. Nur durch die totale Abwesenheit von emulgierenden Substanzen konnte dieser Effekt ausgeschaltet werden.

Substratzubereitungen, die für die Bestimmung von Lipase-, Protease- und Trypsinaktivität eingesetzt wurden, zeigten ausreichende Stabilität, lediglich die Instabilität der Stärke-Lösung führte durchweg zu Störungen der Amylase-Methode. Die erhöhte Temperatur im Vergleich zur Arzneibuchmethode zeigte einen positiven Einfluss auf die Stabilität der Stärkelösung.

Eine Doppelbestimmung einer Enzymaktivität dauert ca.10 min mittels URT und ca. 26 min mit HR-US, was im Unterschied von Zellvolumen und Temperiersystem begründet werden kann. Automatisierbarkeit wurde durch den Gebrauch einer Durchflusszelle (FTS) untersucht. Weil dabei das Substrat-Enzym-Gemisch vor Erreichen der Messzelle automatisch vortemperiert wird, führte der Gebrauch des FTS zu einer Analysenzeitverkürzung. Auch die Übertragung der Vormischung von Substrat und Enzym auf die URT und HR-US-Standgeräte beschleunigt die Analysenzeit, weil die Basislinien-Überprüfung vor Enzymzugabe wegfällt, und verbesserte die Wiederholbarkeit.

Um Ultraschallergebnisse mit denen der Arzneibuchmethoden vergleichen zu können, wurden die Änderungen der Ultraschallgeschwindigkeit von Referenzstandardlösung und Probelösung bestimmt. Die Enzymaktivität wurde dann über die deklarierte Aktivität des Referenzstandards gemäß Arzneibuch berechnet. Für Wirkstoff und Produkt waren die mit HR-US und URT bestimmten Aktivitäten vergleichbar mit den Ergebnissen der Arzneibuchmethoden (Ph.Eur. und USP), was die hohe Selektivität der Ultraschallmethoden bestätigt. Bezüglich Inprozess-Proteolyse-Mustern wurde die Amylase-Methode durch den Einsatz eines höher konzentrierten Puffersystems verbessert. Für die Lipase- und die Protease-Methoden (frei und gesamt) konnte eine gute Anwendbarkeit gezeigt werden. Die Ultraschall-Bestimmung von Trypsin führte jedoch nicht zu verlässlichen Ergebnissen. Durch die hohe Probekonzentration bei dieser Methode ist eine Störung durch die Anwesenheit von Fett und Isopropanol naheliegender als bei den anderen Ultraschallmethoden.
Ein Vergleich der Ultraschallspektroskpie mit alternativen Techniken (NIR/ MIR und Turbidimetrie) wie sie in der Literatur beschrieben sind, zeigt für alle ähnliche Vorteile und Herausforderungen im Hinblick auf die Inprozesskontrolle der Proteolyse.

Zusammenfassend kann festgehalten werden, dass die Ultraschallmessung eine Alternative zu den Arzneibuchmethoden sein kann, um Pankreasenzyme in den meisten Prozessproben, im Wirkstoff und im Produkt zu bestimmen. Um URT oder HR-US im Rahmen von PAT als Inprozesskontrolle zu nutzen, sind noch weitere Experimente zur Automatisierung (online), zur Analysenzeit und zur Ausschaltung von störenden Matrixeffekten zu empfehlen. Gestützt auf die erhobenen Daten, ist die Anwendung von URT und HR-US für Freigabeanalytik für Pankreaspulver und pankreaspulverhaltige Produkte möglich.

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APPENDIX

A I - A III	*Emulsion development*
A IV	*Control of pH during lipase reaction*
A V	*Specificity (general)*
A VI	*Different salts in sample matrix*
A VII	*Isopropanol in sample matrix*
A VIII	*Protein in sample matrix (model: casein)*
A IX	*Fat in sample matrix (model: olive oil)*
A X	*Linearity*
A XI	*Small temperature differences*
A XII	*Wide temperature ranges*
A XIII	*Concentration of substrate*
A XIV	*Stability of substrate preparation (and Lipase: Operating time of Microfluidizer)*
A XV	*Extraction (Dissolving) time*
A XVI	*Lipase: Potential influencing factors of lipase activity depending on substrate emulsion*
A XVII	*Total protease: Activation parameters*
A XVIII	*Holding time in dosing system (titration equipment)*
A XIX	*Amylase: Dextrins as alternative substrates*
A XX	*Proteolysis-like material*
A XXI	*Proteolysis Material I*
A XXII	*Proteolysis Material II*
A XXIII	*Active Pharmaceutical Ingredient (API)*
A XXIV	*Drug products III - VIII*

Appendix A1

Emulsion development

Table A1. Conditions of emulsion preparation using Microfluidizer Fluid Processor M110-EHI. Bile salts (reagents mixture) were added **after** Microfluidization. The behavior of the emulsions in ultrasonic spectrometer were investigated and mean droplet size was determined (Laser diffraction: RI: 1.600/ IZ 15).

No.	Pre emulsification		Microfluidizer			URT	Mean droplet size (nm)		Comments
	Instrument	Conditions	Chambers Pos.1/ Pos. 2	Pressure (bar)	Max. Temp. (°C)	Stable baseline?	1pass	4 pass	
1	Blender (household)	Grade 2/ 15 min	F12Y/ H30Z	690 - 1200	-	No	-	-	-
2			F20Y/ H30Z	1200	-	No	-	-	-
3			H30Z/ F20Y	1200	-	No	-	-	-
			JR20Z/ H30Z	1200	25.0	Only passage 2	397	488	-
6	Hand blender	Grade 6/ 2 min	F20Y/ H30Z	1200	22.9	Only passage 1-2	493	811	-
7	Power mixer with 4 blade propeller stirrer 50/2000	800 rpm/ 15 min	F20Y/ H30Z	1200	20.8	Only passage 1	-	-	Uncompleted pre-emulsification
8					16.9		270	759	
9					22.9		-	-	
10		850 rpm/ 15 min	F20Y/ H30Z	1200	15.8	Only passage 1-3	277	886	
11		950 rpm/ 15 min	F20Y/ H30Z	1200	23.1	Only passage 1-2	260	792	Foam after pre-emulsification
12				900	12.7	Only passages 1-3	428	587	
13				1500	24.8	Only passages 1-2	275	853	
14				1000	23.1		294	801	
15				1100	16.9		347	751	
16				1200	11.7	Only passages 1-3	339	748	Duplicated volume, no foam
17		1050 rpm/ 10 min		1200	14.0		324	569	

Appendix A1I

Emulsion development

Table A2. Conditions of emulsion preparation using Microfluidizer Fluid Processor M110-EHI. Bile salts (reagents mixture) were added **before** Microfluidization. The behavior of the emulsions in ultrasonic spectrometer were investigated and mean droplet size was determined (Laser diffraction: RI: 1.600/ IZ 15).

	Pre emulsification		**Microfluidizer**			**URT**	**Mean droplet size (nm)**		**Comments**
	Instrument	**Conditions**	**Chambers Pos.1/ Pos. 2**	**Pressure (bar)**	**Max. Temp. (°C)**	**Stable baseline?**	**1pass**	**4 pass**	
1	Power mixer with 4 blade propeller stirrer 50/2000	950 rpm/ 15 min	F20Y/ H30Z	1200	12.0	All passages 1-4, high signal after enzyme injection	979	714	Bimodal/ phase separation
2	Blender (household)	grade 2/ 15 min	F20Y/ H30Z	1200	13.0	All passages 1-4	186	159	Dilution with TRIS buffer → increase of initial slopes
3					11.2	All passages 1-12	184	156 (pass 12)	-

Table A3. Pre-emulsion was prepared by adding Reagents mixture (USP/ Ph.Eur.) to the Ph.Eur.-Emulsion. The emulsions were processed using a Microfluidizer at 1200 bar and performing **1 - 6 passages**. Droplet sizes were determined (Laser diffraction, BI: 1.600/ IZ 15). Ultrasonic velocity changes were determined by URT at 37°C adding 4 µl working standard solution (aqueous) to 180µl emulsion (n=2 (1replicate)).

Passage	**1**	**2**	**3**	**4**	**5**	**6**
Temperature after pass. (°C)	11.2	10.7	10.6	10.6	10.5	10.5
Average (µm)	0.184	0.167	0.161	0.160	0.157	0.156
Modal (µm)	0.181	0.162	0.160	0.159	0.158	0.157
Median (µm)	0.179	0.163	0.161	0.157	0.157	0.154
D.o.C. 90%	0.241	0.212	0.201	0.201	0.194	0.193
Velocity change over time ($m \cdot s^{-2}$)	**0.000229**	**0.000221**	**0.000206**	**0.000206**	**0.000199**	**0.000184**
Stdev ($m \cdot s^{-2}$)	0.000008	0.000001	0.000001	0.000001	0.000009	0.000001
CV (%)	**3.7**	**0.2**	**0.2**	**0.3**	**4.5**	**0.2**

Table A4. Pre-emulsion was prepared by adding Reagents mixture to the Ph.Eur.-Emulsion. The emulsions were processed using Microfluidizer at 1200 bar and performing **7 - 12 passages**. Droplet sizes were determined (Laser diffraction, BI: 1.600/ IZ 15). Ultrasonic velocity changes were determined by URT at 37°C adding 4 µl working standard solution (aqueous) to 180µl emulsion (n=2 (1replicate)).

Passage	**7**	**8**	**9**	**10**	**11**	**12**
Temperature after pass. (°C)	10.5	10.5	10.5	10.5	10.4	10.3
Average (µm)	0.160	0.154	0.153	0.158	0.152	0.156
Modal (µm)	0.159	0.157	0.156	0.158	0.156	0.157
Median (µm)	0.157	0.152	0.151	0.154	0.150	0.153
D.o.C. 90%	0.204	0.192	0.191	0.199	0.190	0.197
Velocity change over time ($m \cdot s^{-2}$)	**0.000197**	**0.000192**	**0.000202**	**0.000202**	**0.000191**	**0.000172**
Stdev ($m \cdot s^{-2}$)	0.000023	0.000013	0.000005	0.000010	0.000001	0.000005
CV (%)	**11.9**	**6.8**	**2.2**	**5.0**	**0.3**	**2.7**

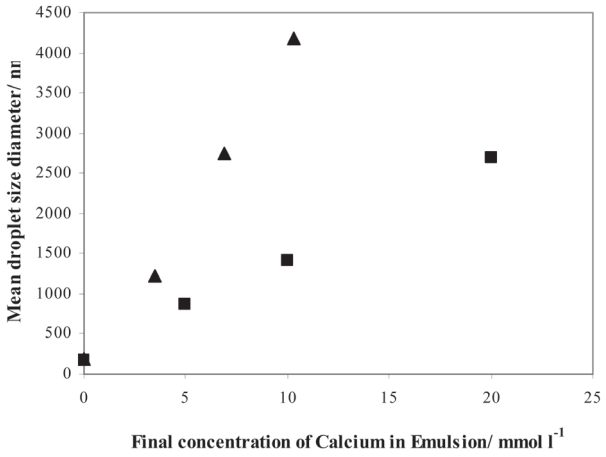

Figure A1. Influence of Calcium on emulsion stability was tested. 25 ml Olive oil were used to prepare the pre emulsion (400 ml). Bile salts were added before high pressure homogenization. The Microfluidizer (MF) was processed at 1200 bar and 3 passages were performed. Shown are the content of calcium in the emulsion and the mean diameter of the droplets determined by using Laser diffraction (IZ 15, BI 1.600). Squares present emulsion preparation when calcium was added after MF, triangles represent calicum addition before MF.

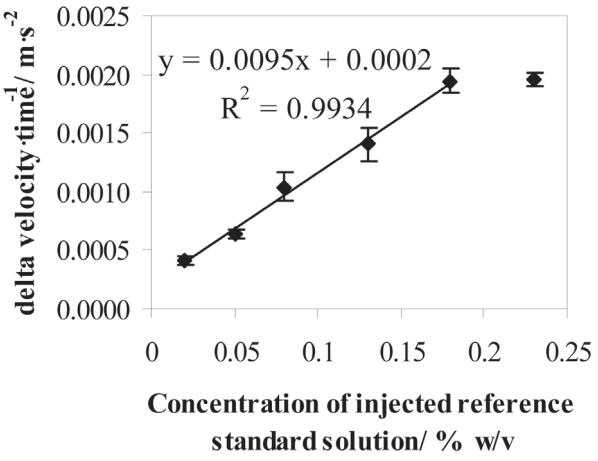

Figure A2. Linearity was determined for inital URT lipase method. Emulsion: 25 ml Olive oil were used to prepare the pre emulsion (400 ml). Bile salts were added before high pressure homogenization./ The Microfluidizer (MF) was processed at 1200 bar and 3 passages were performed. Ultrasonic velocity changes were determined with URT at 37°C adding 4 µl working standard solution (Maleate buffer solution pH 7.5) to 180µl emulsion (n=1 (2 rep), mean +/- stdev).

Table A5. Mean diameter of droplets (Laser diffraction, IZ 15, BI 1.600) and ultrasonic velocity changes during lipase reaction (n=2 (1rep)) were determined in dependence of a various content of olive oil and various settings of microfluidization (pressure, number of passages, time of pre emulsification).

Run	Oil content (ml/ 400 ml)	Pressure (bar)	Number of passages	Time of pre-Emulsification (min)	Change of ultrasonic velocity over time ($m \cdot s^{-2}$)	Droplet size mean (µm)	Temp. (°C)
2	10	600	1	5	0.050171	0.269	13.9
6	10	600	1	15	0.025493	0.262	13.1
5	10	600	5	15	0.026210	0.219	12.7
7	10	600	5	5	0.019111	0.254	12.8
13	10	1800	1	5	0.025808	0.228	13.6
16	10	1800	1	15	0.021156	0.209	16.1
1	10	1800	5	5	0.072356	0.158	13.5
3	10	1800	5	15	0.072901	0.163	13.3
10	25	1200	3	10	0.003711	0.165	13.3
12	25	1200	3	10	0.004416	0.164	15.4
18	25	1200	3	10	0.007650	0.162	14.0
11	40	600	1	5	0.000600	0.239	13.0
17	40	600	1	15	0.000121	0.243	13.9
4	40	600	5	15	0.000115	0.189	12.9
8	40	600	5	5	0.001281	0.194	12.7
14	40	1800	1	5	0.000527	0.186	14.0
19	40	1800	1	15	0.000150	0.183	13.8
15	40	1800	5	15	0.000396	0.145	14.7
9	40	1800	5	5	0.000577	0.151	14.0
23	10	600	1	5	0.048712	0.254	15.0
25	10	600	1	15	0.043204	0.273	14.2
30	10	600	5	5	0.049661	0.241	14.4
36	10	600	5	15	0.042783	0.272	14.1
32	10	1800	1	5	0.020459	0.216	13.8
35	10	1800	1	15	0.025116	0.216	14.3
24	10	1800	5	5	0.073490	0.177	14.6
29	10	1800	5	15	0.039303	0.173	14.8
21	25	1200	3	10	0.009666	0.163	14.1
33	25	1200	3	10	0.006763	0.165	14.3
38	25	1200	3	10	0.007595	0.166	14.7
27	40	600	1	5	0.001012	0.248	14.3
31	40	600	1	15	0.000815	0.240	14.3
28	40	600	5	15	0.001417	0.187	14.1
37	40	600	5	5	0.001420	0.190	14.3
20	40	1800	1	15	0.000394	0.181	14.6
34	40	1800	1	5	0.000224	0.181	14.5
22	40	1800	5	15	0.000447	0.145	14.9
26	40	1800	5	5	0.001331	0.153	14.7

Appendix A1II

Emulsion development

Table A6. Ultrasonic velocity change during lipase reaction (URT) and modal droplet sizes of the used emulsion were determined shown according to the number of passages in Microfluidizer.

Number of passages	**1**	**2**	**3**	**4**
Std Work 1 ($m \cdot s^{-2}$)	0.000763	0.000888	0.000756	0.000800
Std Work 2 ($m \cdot s^{-2}$)	0.000793	0.000741	0.000771	0.000764
Mean ($m \cdot s^{-2}$)	**0.000778**	**0.000815**	**0.000764**	**0.000782**
Stdev ($m \cdot s^{-2}$)	0.000021	0.000104	0.000010	0.000025
CV (%)	**2.7**	**12.8**	**1.4**	**3.2**
Modal droplet size (µm)	0.105	0.094	0.093	0.092

Table A7. Ultrasonic velocity change during lipase reaction (URT) and modal droplet sizes of the used emulsion were determined shown according to the olive oil concentration in emulsion.

c (olive oil) in pre-emulsion (% (v/v))	**1**	**2**	**4**	**6**
c (olive oil) in final emulsion (% (v/v))	0.5	1	2	3
Std Work 1 ($m \cdot s^{-2}$)	0.000662	0.000856	0.000993	0.000943
Std Work 2 ($m \cdot s^{-2}$)	0.000653	0.000904	-	-
Mean ($m \cdot s^{-2}$)	**0.000658**	**0.000880**	**0.000993**	**0.000943**
Stdev ($m \cdot s^{-2}$)	0.000062	0.000034	-	-
CV (%)	**1.0**	**3.9**	**-**	**-**
Modal droplet size in final emulsion (µm)	0.140	0.094	0.107	0.127

Table A8. Ultrasonic velocity change during lipase reaction (URT) and modal droplet sizes of the used emulsion was determined shown according to the Triton X 100 concentration in emulsion.

c (Triton X 100) in pre-emulsion ($mmol \cdot l^{-1}$)	**1**	**3**	**5**	**7**
c (Triton X 100) in final emulsion ($mmol \cdot l^{-1}$)	0.5	1.5	2.5	3.5
Std Work 1 ($m \cdot s^{-2}$)	-	0.001112	0.001000	0.000949
Std Work 2 ($m \cdot s^{-2}$)	-	0.001153	0.000942	0.000956
Mean ($m \cdot s^{-2}$)	**-**	**0.001133**	**0.000971**	**0.000952**
Stdev ($m \cdot s^{-2}$)	-	0.000029	0.000041	0.000052
CV (%)	**-**	**2.6**	**4.2**	**0.6**
Modal droplet size in final emulsion modal (µm)	0.541	0.184	0.138	0.111
average droplet size in final emulsion (µm)	0.530	0.214	0.153	0.122

Table A9. Ultrasonic velocity change during lipase reaction (URT) and modal droplet sizes of the used emulsion were determined shown according to the $CaCl_2$ concentration in emulsion (1. part).

c ($CaCl_2$) in pre-emulsion (mmol·l^{-1})	**80**	**160**	**240**	**320**	**400**
c ($CaCl_2$) in final emulsion (mmol·l^{-1})	40	80	120	160	200
Std Work 1 (m·s^{-2})	0.001312	0.001366	0.001464	0.001402	0.001286
Std Work 2 (m·s^{-2})	0.001245	0.001186	0.001407	0.001487	0.001282
Mean (m·s^{-2})	**0.001278**	**0.001276**	**0.001435**	**0.001445**	**0.001284**
Stdev (m·s^{-2})	0.000047	0.000128	0.000040	0.000060	0.000003
CV (%)	**3.7**	**10.0**	**2.8**	**4.2**	**0.3**
Modal droplet size in final emulsion modal (μm)	0.1254	0.1262	0.1276	0.1343	0.1253

Table A10. Ultrasonic velocity change during lipase reaction (URT) and modal droplet sizes of the used emulsion were determined shown according to the $CaCl_2$ concentration in emulsion (2. part).

c ($CaCl_2$) in pre-emulsion (mmol·l^{-1})	**5**	**10**	**20**	**40**	**80**
c ($CaCl_2$) in final emulsion (mmol·l^{-1})	2.5	5	10	20	40
Std Work 1 (m·s^{-2})	0.000554	0.000815	0.000935	0.001047	0.001281
Std Work 2 (m·s^{-2})	0.000535	0.000848	0.001041	0.001076	0.001302
Mean (m·s^{-2})	**0.000544**	**0.000832**	**0.000988**	**0.001062**	**0.001291**
Stdev (m·s^{-2})	0.000014	0.000023	0.000074	0.000020	0.000015
CV (%)	**2.5**	**2.8**	**7.5**	**1.9**	**1.2**
Modal droplet size in final emulsion modal (μm)	0.1346	0.1259	0.1341	0.1272	0.1348

Table A11. Ultrasonic velocity change during lipase reaction (URT) and modal droplet sizes of the used emulsion were determined shown according to the TRIS concentration in emulsion.

c (TRIS) in pre-emulsion (mmol·l^{-1})	**20**	**80**	**100**	**120**	**160**
c (TRIS) in final emulsion (mmol·l^{-1})	10	40	50	60	80
Std Work 1 (m·s^{-2})	0.001125	0.001333	0.001397	0.001332	0.001496
Std Work 2 (m·s^{-2})	0.001136	0.001341	0.001455	0.001280	0.001388
Mean (m·s^{-2})	**0.001131**	**0.001337**	**0.001426**	**0.001306**	**0.001442**
Stdev (m·s^{-2})	0.000007	0.000006	0.000041	0.000037	0.000076
CV (%)	**0.7**	**0.5**	**2.9**	**2.8**	**5.3**
Modal droplet size in final emulsion modal (μm)	0.1269	0.1247	0.1353	0.1273	0.1259

Appendix A IV

Control of pH during lipase reaction

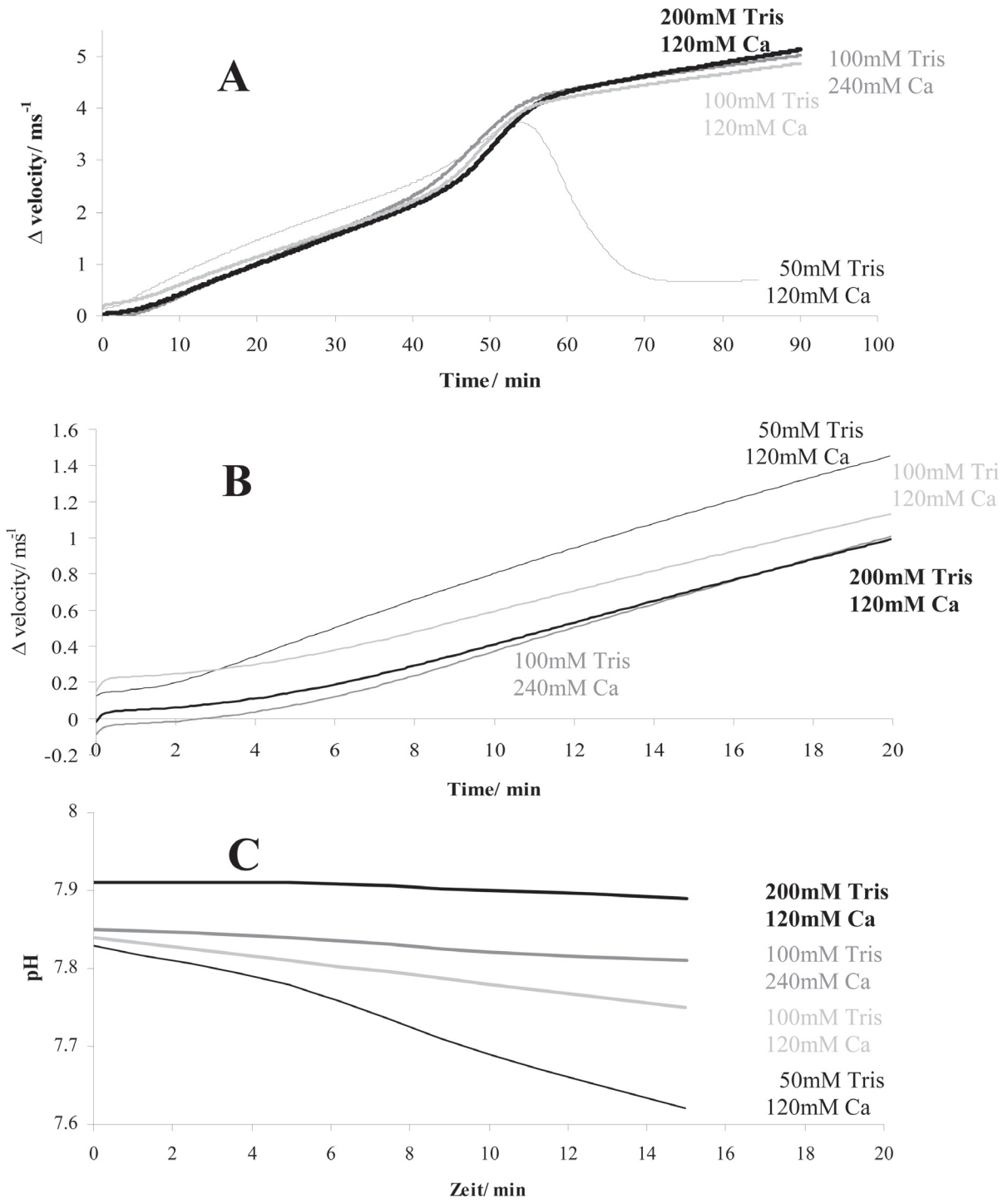

Figure A3. **A:** The change of ultrasonic velocity over time during lipase reaction was determined using URT and four buffer compositions (pH 7.8). Using the 'standard' emulsion a strong decrease of ultrasonic velocity after 55 minutes was observed that correlated with a strong coalescence of the olive oil droplets monitored with the micrcoscope. Using the other emulsions similar courses of the kinetic curves were not observed in the tested time period. **B (Detail of A):** The change of ultrasonic velocity over time (0 - 20 min) during lipase reaction was determined using URT and four buffer compositions (pH 7.8).The emulsion with lowest ionic concentration shows the strongest increase of ultrasonic velocity. **C:** The change of pH over time during lipase reaction was monitored using four buffer compositions (pH 7.8). The pH decreased over time. Decrease of pH during enzyme reaction was minimized by increasing the TRIS concentration. Coalescence was also reduced at higher TRIS concentrations (microscope). Concerning the results, a strong interference was presumed between degradation of olive oil on the one hand and droplet coalescence on the other hand when using the low concentrated buffer system (Tris 50mM).

Appendix A V

Specificity (general)

Table A12. Specificity test for **amylase** activity determination by ultrasonic method (URT). In the complete system (positive control), ultrasonic velocity was measured at 37°C after adding 4µl of Amylase solution (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US). On the left, the system lacked pancreas powder, on the right, the system lacked starch. Shown are means of 2 replicates per working standard solution.

	Starch solution 6% (URT/ HR-US) + Phosphate buffer solution (URT/ HR-US)	**Starch solution 6% (URT/ HR-US) + Amylase solution (URT/ HR-US)**	**Phosphate buffer solution (URT/ HR-US) + Amylase solution (URT/ HR-US)**
Std Work 1 ($m \cdot s^{-2}$)	-0.000016	0.001226	0.000010
Std Work 2 ($m \cdot s^{-2}$)	-0.000069	0.001261	-0.000003
Mean ($m \cdot s^{-2}$)	**-0.000043**	**0.001244**	**0.000004**
Stdv ($m \cdot s^{-2}$)	0.000037	0.000025	0.000010
CV (%)	**87.2**	**2.0**	**277.6**

Table A13. Specificity test for **lipase** activity determination by ultrasonic method (HR-US). In the complete system (positive control), ultrasonic velocity was measured at 37°C after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US). On the left, the system lacked pancreas powder, on the right, the system lacked olive oil. Shown are means of 2 replicates per working standard solution.

	Olive oil emulsion (URT/ HR-US) + Maleate buffer solution pH 7.0 (URT/ HR-US)	**Olive oil emulsion (URT/ HR-US) + Lipase Solution (URT/ HR-US)**	**Bile salts solution (URT/ HR-US)/ TRIS buffer solution (URT/ HR-US) + Lipase Solution (URT/ HR-US)**
Std Work 1 ($m \cdot s^{-2}$)	-0.000009	0.005742	0.000003
Std Work 2 ($m \cdot s^{-2}$)	-0.000010	0.005845	-0.000014
Mean ($m \cdot s^{-2}$)	**-0.000010**	**0.005793**	**-0.000005**
Stdv ($m \cdot s^{-2}$)	0.000000	0.000072	0.000012
CV (%)	**4.3**	**1.3**	**227.5**

Table A14. Specificity test for **free protease** activity determination by ultrasonic method (URT). In the complete system (positive control), ultrasonic velocity was measured at 37°C after adding 4 µl of 0.5 % Std Work solution (aqueous) to 180 µl of casein 0.625 % (URT/ HR-US). On the left, the system lacked pancreas powder, on the right, the system lacked casein. Shown are means of 2 replicates per working standard solution.

	Casein solution 0.625 % (URT/ HR-US) + water	**Casein solution 0.625 % (URT/ HR-US) + Std Work (in water)**	**Water + Std Work (in water)**
Std Work 1 ($m \cdot s^{-2}$)	0.000010	0.001067	-0.000005
Std Work 2 ($m \cdot s^{-2}$)	0.000004	0.001109	-0.000001
Mean ($m \cdot s^{-2}$)	0.000007	0.001088	-0.000003
Stdv ($m \cdot s^{-2}$)	0.000005	0.000030	0.000003
CV (%)	67.1	2.7	95.9

Table A15. Specificity test for **total protease** activity determination by ultrasonic method (URT). In the complete system (positive control), ultrasonic velocity was measured at 37°C using a premix of 1800 µl casein 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase. On the left, the system lacked pancreas powder, on the right, the system lacked casein. Shown are means of 2 replicates per working standard solution.

	Casein solution 1.25 % + $CaCl_2$ solution 0.02 M (URT/ HR-US)	**Casein solution 1.25% + Total protease solution (URT/ HR-US)**	**Water + Total protease solution (URT/ HR-US)**
Std Work 1 ($m \cdot s^{-2}$)	0.000049	0.000962	-0.000015
Std Work 2 ($m \cdot s^{-2}$)	0.000045	0.001001	-0.000017
Mean ($m \cdot s^{-2}$)	**0.000047**	**0.000982**	**-0.000016**
Stdv ($m \cdot s^{-2}$)	0.000003	0.000027	0.000002
CV (%)	**5.6**	**2.8**	**10.8**

Table A16. Specificity test for **free trypsin** activity determination by ultrasonic method (URT). In the complete system (positive control), ultrasonic velocity was measured at 37°C after adding 8 µl of trypsin solution (URT/ HR-US) to 180 µl of BAEE solution (URT/ HR-US). On the left, the system lacked pancreas powder and Chymotrypsin was used, respectively, on the right, the system lacked BAEE. Shown are means of 2 replicates per working standard solution.

	BAEE solution (URT/ HR-US) + Triton X 100 solution (URT/ HR-US)	**BAEE solution (URT/ HR-US) + Chymotrypsin (in Triton X 100 solution (URT/ HR-US))**	**BAEE solution (URT/ HR-US) + Trypsin solution (URT/ HR-US)**	**TRIS buffer (URT/ HR-US) + Trypsin solution (URT/ HR-US)**
Std Work 1 ($m \cdot s^{-2}$)	-0.000001	0.000005	0.000875	0.000006
Std Work 2 ($m \cdot s^{-2}$)	0.000002	0.000001	0.000869	-0.000003
Mean ($m \cdot s^{-2}$)	**0.000001**	**0.000003**	**0.000872**	**0.000002**
Stdv ($m \cdot s^{-2}$)	0.000002	0.000003	0.000005	0.000006
CV (%)	**418.9**	**89.4**	**0.5**	**412.2**

Appendix A VI

Different salts in sample matrix

Table A17. Influence of NaCl concentration in sample matrix on ultrasonic determination of **amylase** activity (URT). Amylase solutions (URT/ HR-US) with different NaCl concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 4µl of Amylase solution (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

NaCl in solvent ($mmol \cdot l^{-1}$)	**0**	**3.35**	**6.7**	**10.05**	**13.4**	**26.8**	**53.6**
Std Work 1 ($m \cdot s^{-2}$)	0.001282	0.001246	0.001271	0.001320	0.001304	0.001344	0.001294
Std Work 2 ($m \cdot s^{-2}$)	0.001270	0.001280	0.001276	0.001293	0.001280	0.001262	0.001285
Mean ($m \cdot s^{-2}$)	0.001276	0.001263	0.001273	0.001307	0.001292	0.001303	0.001290
Stdv ($m \cdot s^{-2}$)	0.000009	0.000025	0.000003	0.000019	0.000017	0.000058	0.000006
CV (%)	**0.7**	**1.9**	**0.3**	**1.5**	**1.3**	**4.4**	**0.5**

Table A18. Influence of NaCl concentration in sample matrix on ultrasonic determination of **lipase** activity (HR-US). Lipase solutions (URT/ HR-US) with different NaCl concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 20µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

NaCl in sample solvent ($mmol \cdot l^{-1}$)	**0**	**86**	**171**	**257**	**342**
Std Work 1 ($m \cdot s^{-2}$)	0.005698	0.005719	0.005792	0.005806	0.005869
Std Work 2 ($m \cdot s^{-2}$)	0.005723	0.005706	0.005735	0.005777	0.005944
Mean ($m \cdot s^{-2}$)	**0.005710**	**0.005712**	**0.005763**	**0.005791**	**0.005907**
Stdv ($m \cdot s^{-2}$)	0.000017	0.000009	0.000041	0.000020	0.000053
CV (%)	**0.3**	**0.2**	**0.7**	**0.4**	**0.9**

Table A19. Influence of $CaCl_2$ concentration in sample matrix on ultrasonic determination of **amylase** activity (URT). Amylase solutions (URT/ HR-US) with different $CaCl_2$ concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 4µl of Amylase solution (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

$CaCl_2$ in sample solvent ($mmol \cdot l^{-1}$)	**0**	**0.88**	**3.35**
Std Work 1 ($m \cdot s^{-2}$)	0.001038	0.001057	0.001067
Std Work 2 ($m \cdot s^{-2}$)	0.000989	0.000975	0.001050
Mean ($m \cdot s^{-2}$)	**0.001013**	**0.001016**	**0.001059**
Stdv ($m \cdot s^{-2}$)	0.000035	0.000057	0.000012
CV (%)	**3.4**	**5.7**	**1.2**

Table A20. Influence of $CaCl_2$ concentration in sample matrix on ultrasonic determination of **lipase** activity (HR-US). Lipase solutions (URT/ HR-US) with different $CaCl_2$ concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 20µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

$CaCl_2$ in sample solvent ($mmol \cdot l^{-1}$)	**0**	**0.001**	**1.0**	**10**	**100**	**1000**
Std Work 1 ($m \cdot s^{-2}$)	0.005692	0.005798	0.005680	0.005687	0.005715	0.005886
Std Work 2 ($m \cdot s^{-2}$)	0.005672	0.005651	0.005665	0.005683	0.005801	0.005776
Mean ($m \cdot s^{-2}$)	**0.005682**	**0.005724**	**0.005672**	**0.005685**	**0.005758**	**0.005831**
Stdv ($m \cdot s^{-2}$)	0.000014	0.000104	0.000011	0.000003	0.000061	0.000078
CV (%)	**0.3**	**1.8**	**0.2**	**0.1**	**1.1**	**1.3**

Table A21. Influence of $CaCl_2$ concentration in sample matrix on ultrasonic determination of **total protease** activity (URT). Total protease solutions (URT/ HR-US) with different $CaCl_2$ concentrations were prepared. Ultrasonic velocity was measured at 37°C using a premix of 1800 µl casein 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase. Shown are means of 2 replicates per working standard solution.

$CaCl_2$ in sample solvent ($mmol \cdot l^{-1}$)	**0.0**	**0.8**	**10.2**	**20.0**
Std Work 1 ($m \cdot s^{-2}$)	0.001004	0.001067	0.000996	0.001024
Std Work 2 ($m \cdot s^{-2}$)	0.000998	0.000999	0.001006	0.000984
Mean ($m \cdot s^{-2}$)	**0.001001**	**0.001033**	**0.001001**	**0.001004**
Stdv ($m \cdot s^{-2}$)	0.000005	0.000048	0.000008	0.000029
CV (%)	**0.5**	**4.6**	**0.8**	**2.9**

Table A22. Influence of $CaCl_2$ concentration in sample matrix on ultrasonic determination of **free trypsin** activity (HR-US). Trypsin solutions (URT/ HR-US) with different $CaCl_2$ concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 20 µl of Trypsin solution (URT/ HR-US) to 1050 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

$CaCl_2$ in sample solvent ($mmol \cdot l^{-1}$)	**0**	**100**	**200**	**300**
Std Work 1 ($m \cdot s^{-2}$)	0.000394	0.000375	0.000398	0.000390
Std Work 2 ($m \cdot s^{-2}$)	0.000360	0.000353	0.000355	0.000348
Mean ($m \cdot s^{-2}$)	**0.000377**	**0.000364**	**0.000376**	**0.000369**
Stdv ($m \cdot s^{-2}$)	0.000024	0.000015	0.000031	0.000030
CV (%)	**6.5**	**4.2**	**8.2**	**8.0**

Appendix A VII

Isopropanol in sample matrix

Table A23. Influence of isopropanol concentration in sample matrix on ultrasonic determination of **amylase** activity (URT). Amylase solutions (URT/ HR-US) with different isopropanol concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 4µl of Amylase solution (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Isopropanol in sample solvent (% (v/v))	**0**	**10**	**20**	**25**	**30**
Isopropanol in sample solvent (% (w/w))	0	8	16	20	25
Std Work 1 ($m \cdot s^{-2}$)	0.001311	0.001363	0.001244	0.001313	0.001306
Std Work 2 ($m \cdot s^{-2}$)	0.001290	0.001271	0.001251	-	0.001299
Mean ($m \cdot s^{-2}$)	**0.001301**	**0.001317**	**0.001247**	**0.001313**	**0.001302**
Stdv ($m \cdot s^{-2}$)	0.000015	0.000066	0.000004	-	0.000005
CV (%)	**1.2**	**5.0**	**0.4**	**-**	**0.4**

Table A24. Influence of isopropanol concentration in sample matrix on ultrasonic determination of **lipase** activity (HR-US). Lipase solutions (URT/ HR-US) with different isopropanol concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 20µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Isopropanol in sample solvent (% (v/v))	**0**	**10**	**20**	**25**	**30**
Isopropanol in sample solvent (% (w/w))	0	8	16	20	25
Std Work 1 ($m \cdot s^{-2}$)	0.005765	0.005793	0.005804	0.005696	0.005737
Std Work 2 ($m \cdot s^{-2}$)	0.005990	0.005886	0.005878	0.005516	-
Mean ($m \cdot s^{-2}$)	**0.005878**	**0.005840**	**0.005841**	**0.005606**	**0.005737**
Stdv ($m \cdot s^{-2}$)	0.000159	0.000066	0.000052	0.000128	-
CV (%)	**2.7**	**1.1**	**0.9**	**2.3**	**-**

Table A25. Influence of isopropanol concentration in sample matrix on ultrasonic determination of **free protease** activity (URT). Free protease solutions (URT/ HR-US) with different isopropanol concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 8 µl of Trypsin solution (URT/ HR-US) to 180 µl of Casein solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Isopropanol in sample solvent (% (v/v))	**0**	**10**	**20**	**25**	**30**
Isopropanol in sample solvent (% (w/w))	0	8	16	20	25
Std Work 1 ($m \cdot s^{-2}$)	0.001127	0.001060	0.001049	0.001024	0.001063
Std Work 2 ($m \cdot s^{-2}$)	0.001105	0.001062	0.001093	0.001039	0.001075
Mean ($m \cdot s^{-2}$)	**0.001116**	**0.001061**	**0.001071**	**0.001032**	**0.001069**
Stdv ($m \cdot s^{-2}$)	0.000016	0.000002	0.000031	0.000010	0.000008
CV (%)	**1.4**	**0.1**	**2.9**	**1.0**	**0.8**

Table A26. Influence of isopropanol concentration in sample matrix on ultrasonic determination of **total protease** activity (URT). Total protease solutions (URT/ HR-US) with different isopropanol concentrations were prepared. Ultrasonic velocity was measured at 37°C using a premix of 1800 µl casein 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase.

Isopropanol in sample solvent (% (v/v))	**0**	**10**	**20**	**25**	**30**
Isopropanol in sample solvent (% (w/w))	0	8	16	20	25
Std Work 1 ($m \cdot s^{-2}$)	0.000962	0.000951	0.000958	0.000967	0.000890
Std Work 2 ($m \cdot s^{-2}$)	0.001001	0.000952	0.000959	0.000937	0.000943
Mean ($m \cdot s^{-2}$)	**0.000982**	**0.000951**	**0.000958**	**0.000952**	**0.000917**
Stdv ($m \cdot s^{-2}$)	0.000027	0.000001	0.000000	0.000021	0.000037
CV (%)	**2.8**	**0.1**	**0.0**	**2.3**	**4.1**

Table A27. Influence of isopropanol concentration in sample matrix on ultrasonic determination of **free trypsin** activity (URT). Trypsin solutions (URT/ HR-US) with different isopropanol concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 8 µl of Trypsin solution (URT/ HR-US) to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Isopropanol in sample solvent (% (v/v))	**0**	**10**	**20**	**25**	**30**
Isopropanol in sample solvent (% (w/w))	0	8	16	20	25
Std Work 1 ($m \cdot s^{-2}$)	0.000885	0.000848	0.000917	0.000898	0.000951
Std Work 2 ($m \cdot s^{-2}$)	0.000827	0.000865	0.001070	0.001138	0.001046
Mean ($m \cdot s^{-2}$)	**0.000856**	**0.000857**	**0.000993**	**0.001018**	**0.000999**
Stdv ($m \cdot s^{-2}$)	0.000041	0.000012	0.000108	0.000170	0.000067
CV (%)	**4.8**	**1.4**	**10.9**	**16.7**	**6.7**

Appendix A VIII

Protein in sample matrix (model: casein)

Table A28. Influence of protein concentration in sample matrix on ultrasonic determination of **amylase** activity (URT). Amylase solutions (URT/ HR-US) with different casein concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 4µl of Amylase solution (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

c Casein (% (w/v))	**0**	**0.31**	**0.63**	**0.94**	**1.25**
Std Work 1 ($m \cdot s^{-2}$)	0.001211	0.001201	0.001285	0.001318	0.001286
Std Work 2 ($m \cdot s^{-2}$)	0.001187	0.001289	0.001279	0.001273	0.001293
Mean ($m \cdot s^{-2}$)	0.001199	0.001245	0.001282	0.001295	0.001290
Stdv ($m \cdot s^{-2}$)	0.000017	0.000062	0.000004	0.000031	0.000005
CV (%)	1.4	5.0	0.3	2.4	0.4

Table A29. Influence of protein concentration in sample matrix on ultrasonic determination of **amylase** activity (URT). Amylase solutions 0.2 M (URT/ HR-US) with different casein concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 4µl of Amylase solution 0.2 M (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

c Casein (% (w/v))	**0**	**0.31**	**0.63**	**1.25**
Std Work 1 ($m \cdot s^{-2}$)	0.001231	0.001257	0.001288	0.001290
Std Work 2 ($m \cdot s^{-2}$)	0.001261	0.001260	0.001251	0.001269
Mean ($m \cdot s^{-2}$)	0.001246	0.001258	0.001269	0.001280
Stdv ($m \cdot s^{-2}$)	0.000021	0.000002	0.000027	0.000015
CV (%)	1.7	0.2	2.1	1.2

Table A30. Influence of protein concentration in sample matrix on ultrasonic determination of **lipase** activity (HR-US). Lipase solutions (URT/ HR-US) with different casein concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 20µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

c Casein (% (w/v))	**0**	**0.31**	**0.63**	**0.94**	**1.25**
Std Work 1 ($m \cdot s^{-2}$)	0.006849	0.006670	0.006877	0.006807	0.006693
Std Work 2 ($m \cdot s^{-2}$)	0.006786	0.006712	0.006840	0.006795	0.006785
Mean ($m \cdot s^{-2}$)	0.006817	0.006691	0.006859	0.006801	0.006739
Stdv ($m \cdot s^{-2}$)	0.000044	0.000030	0.000026	0.000009	0.000065
CV (%)	0.6	0.4	0.4	0.1	1.0

Table A31. Influence of protein concentration in sample matrix on ultrasonic determination of **free trypsin** activity (URT). Trypsin solutions (URT/ HR-US) with different casein concentrations were prepared. Ultrasonic velocity was measured at 37°C after adding 8 µl of Trypsin solution (URT/ HR-US) to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

c Casein (% (w/v))	**0**	**0.31**	**0.63**	**0.94**	**1.25**
Std Work 1 ($m \cdot s^{-2}$)	0.000814	0.000808	0.000805	0.000818	0.000829
Std Work 2 ($m \cdot s^{-2}$)	0.000814	0.000790	0.000794	0.000811	0.000806
Mean ($m \cdot s^{-2}$)	**0.000814**	**0.000799**	**0.000800**	**0.000814**	**0.000817**
Stdv ($m \cdot s^{-2}$)	0.000000	0.000012	0.000007	0.000005	0.000016
CV (%)	**0.0**	**1.5**	**0.9**	**0.6**	**2.0**

Appendix A IX

Fat in sample matrix (model: olive oil)

Table A32. Influence of fat concentration in sample matrix on ultrasonic determination of **amylase** activity (URT). Phosphate buffer solutions (URT/ HR-US) with different olive oil content, which were used to prepare different Amylase solutions (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 4 µl of Amylase solution (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0.0**	**0.3**	**0.5**	**1.0**	**2.0**	**2.5**	**5.0**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	0.001324	0.001371	0.001223	0.001068	0.000867	0.001176	0.000821	0.000802
Std Work 2 ($m \cdot s^{-2}$)	0.001346	0.001282	0.001245	0.001128	0.000909	0.001015	0.000937	0.001114
Mean ($m \cdot s^{-2}$)	**0.001335**	**0.001327**	**0.001234**	**0.001098**	**0.000888**	**0.001096**	**0.000879**	**0.000958**
Stdv ($m \cdot s^{-2}$)	0.000016	0.000063	0.000016	0.000042	0.000030	0.000114	0.000082	0.000220
CV (%)	**1.2**	**4.8**	**1.3**	**3.9**	**3.4**	**10.4**	**9.3**	**23.0**

Table A33. Influence of fat concentration in sample matrix on ultrasonic determination of **lipase** activity (HR-US). Maleate buffer solutions pH 7.0 (URT/ HR-US) with different olive oil content, which were used to prepare different Lipase solutions (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0**	**2.5**	**5**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	0.005920	0.005841	0.005545	0.005656
Std Work 2 ($m \cdot s^{-2}$)	0.005762	0.005731	0.005692	0.005699
Mean ($m \cdot s^{-2}$)	**0.005841**	**0.005786**	**0.005619**	**0.005678**
Stdv ($m \cdot s^{-2}$)	0.000112	0.000078	0.000104	0.000030
CV (%)	**1.9**	**1.3**	**1.8**	**0.5**

Table A34. Influence of fat concentration in sample matrix on ultrasonic determination of **free protease** activity (URT). Borate buffers pH 7.5 (URT/ HR-US) with different olive oil content, which were used to prepare different Free protease solutions (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 4 µl of Free protease solution (URT/ HR-US) to 180 µl of Casein 1.25 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0.0**	**2.5**	**5.0**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	0.001106	0.001120	0.001179	0.001260
Std Work 2 ($m \cdot s^{-2}$)	0.001114	0.001148	0.001138	0.001250
Mean ($m \cdot s^{-2}$)	**0.001110**	**0.001134**	**0.001159**	**0.001255**
Stdv ($m \cdot s^{-2}$)	0.000006	0.000020	0.000030	0.000007
CV (%)	**0.5**	**1.8**	**2.6**	**0.6**

Table A35. Influence of fat concentration in sample matrix on ultrasonic determination of **total protease** activity (URT). Calcium chloride solutions 0.02 M (URT/ HR-US) with different olive oil content, which were used to prepare different Total protease solutions (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C using a premix of 1800 µl casein 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase. Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0**	**2.5**	**5**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	0.001043	0.001026	0.001029	0.001014
Std Work 2 ($m \cdot s^{-2}$)	0.000997	0.000979	0.001024	0.001027
Mean ($m \cdot s^{-2}$)	**0.001020**	**0.001003**	**0.001027**	**0.001021**
Stdv ($m \cdot s^{-2}$)	0.000032	0.000034	0.000003	0.000009
CV (%)	**3.2**	**3.3**	**0.3**	**0.9**

Table A36. Influence of fat concentration in sample matrix on ultrasonic determination of **free trypsin** activity (URT). Triton X 100 solutions (URT/ HR-US) with different olive oil content, which were used to prepare different Trypsin solutions (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 4 µl of Trypsin solution (URT/ HR-US) to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0.0**	**2.5**	**5.0**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	0.000909	0.001266	0.001597	0.001790
Std Work 2 ($m \cdot s^{-2}$)	0.000859	0.001236	0.001676	0.001836
Mean ($m \cdot s^{-2}$)	**0.000884**	**0.001251**	**0.001637**	**0.001813**
Stdv ($m \cdot s^{-2}$)	0.000036	0.000021	0.000056	0.000033
CV (%)	**4.0**	**1.7**	**3.4**	**1.8**

Table A37. Influence of fat concentration in sample matrix on ultrasonic determination of **free trypsin** activity (URT). Triton X 100 solutions (URT/ HR-US) with different olive oil content, which were used to prepare different Trypsin solutions (0.031 % Std FIP in Triton solution (URT/ HR-US)), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 4 µl of the Trypsin solution to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0.0**	**2.5**	**5.0**	**7.5**	**7.5 % (v/v) water**
Std FIP 1 ($m \cdot s^{-2}$)	0.000842	0.000860	0.001454	0.003212	0.000825
Std FIP 2 ($m \cdot s^{-2}$)	0.000800	0.000816	0.001202	0.001972	0.000849
Mean ($m \cdot s^{-2}$)	**0.000821**	**0.000838**	**0.001328**	**0.002592**	**0.000837**
Stdv ($m \cdot s^{-2}$)	0.000029	0.000032	0.000178	0.000877	0.000017
CV (%)	**3.6**	**3.8**	**13.4**	**33.8**	**2.0**

Fat in sample matrix (model: olive oil) - Additional tests

Amylase: Blanks

Table A38. Influence of fat concentration in sample matrix on ultrasonic determination of **amylase** activity (URT) without starch (blanks). Phosphate buffer solutions (URT/ HR-US) with different olive oil content, which were used to prepare different Amylase solutions (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 4 µl of Amylase solution (URT/ HR-US) to 180 µl of Phosphate buffer solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0**	**2.5**	**5**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	- 0.000002	0.000055	0.000148	0.000238
Std Work 2 ($m \cdot s^{-2}$)	- 0.000006	0.000061	0.000157	0.000188
Mean ($m \cdot s^{-2}$)	**- 0.000004**	**0.000058**	**0.000153**	**0.000213**
Stdv ($m \cdot s^{-2}$)	0.000002	0.000005	0.000006	0.000036
CV (%)	**60.8**	**7.9**	**4.2**	**16.7**

Amylase: Preparation of starch and enzyme solution in 0.2 M phosphate buffer

Table A39. Influence of fat concentration in sample matrix on ultrasonic determination of **amylase** activity (HR-US) using Phosphate buffer solution 0.2 M for preparation of starch and enzyme solution. Phosphate buffer solutions 0.2 M (URT/ HR-US) with different olive oil content, which were used to prepare different Amylase solutions 0.2 M (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 20 µl of Amylase solution 0.2 M (URT/ HR-US) to 1050 µl of Starch solution 6 % (URT/ HR-US modified, prepared in Phosphate buffer solution 0.2 M). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0**	**2.5**	**5**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	0.000962	0.000478	0.000410	0.000806
Std Work 2 ($m \cdot s^{-2}$)	0.000627	0.000792	0.000864	0.000794
Mean ($m \cdot s^{-2}$)	**0.000794**	**0.000635**	**0.000637**	**0.000800**
Stdv ($m \cdot s^{-2}$)	0.000237	0.000222	0.000321	0.000008
CV (%)	**29.8**	**35.0**	**50.4**	**1.0**

Table A40. Influence of fat concentration in sample matrix on ultrasonic determination of **amylase** activity (URT) using Phosphate buffer solution 0.2 M for preparation of starch and enzyme solution. Phosphate buffer solutions 0.2 M (URT/ HR-US) with different olive oil content, which were used to prepare different Amylase solutions 0.2 M (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 4 µl of Amylase solution 0.2 M (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US modified, prepared in Phosphate buffer solution 0.2 M). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0**	**2.5**	**5**
Std Work 1 ($m \cdot s^{-2}$)	0.000994	0.001211	0.001344
Std Work 2 ($m \cdot s^{-2}$)	0.001148	0.000978	0.001117
Mean ($m \cdot s^{-2}$)	**0.001071**	**0.001094**	**0.001231**
Stdv ($m \cdot s^{-2}$)	0.000109	0.000165	0.000160
CV (%)	**10.1**	**15.1**	**13.0**

Amylase: Preparation of enzyme solution in 0.2 M phosphate buffer

Table A41. Influence of fat concentration in sample matrix on ultrasonic determination of **amylase** activity (URT) using Phosphate buffer solution 0.2 M for preparation of enzyme solution. Phosphate buffer solutions 0.2 M (URT/ HR-US) with different olive oil content, which were used to prepare different Amylase solutions 0.2 M (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C using a premix of 1800 µl Starch solution 3 % (URT/ HR-US) and 40 µl Amylase solution 0.2 M (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0**	**2.5**	**5**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	0.001241	0.001293	0.001267	0.001258
Std Work 2 ($m \cdot s^{-2}$)	0.001232	0.001241	0.001256	0.001219
Mean ($m \cdot s^{-2}$)	**0.001237**	**0.001267**	**0.001262**	**0.001238**
Stdv ($m \cdot s^{-2}$)	0.000006	0.000036	0.000008	0.000028
CV (%)	**0.5**	**2.9**	**0.6**	**2.2**

Total protease: Presence of Emulsifier (Triton X 100)

Table A42. Influence of fat concentration in sample matrix on ultrasonic determination of **total protease** activity (URT) in the presence of emulsifier. Calcium chloride solutions 0.02 M (URT/ HR-US) with different olive oil content in the presence of Triton X 100, which were used to prepare different Total protease solutions (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C using a premix of 1800 µl casein 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase. Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0**	**2.5**	**5**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	0.001056	0.000998	0.001020	0.001076
Std Work 2 ($m \cdot s^{-2}$)	0.001045	0.001057	0.001035	0.001081
Mean ($m \cdot s^{-2}$)	**0.001051**	**0.001027**	**0.001027**	**0.001079**
Stdv ($m \cdot s^{-2}$)	0.000007	0.000042	0.000010	0.000004
CV (%)	**0.7**	**4.1**	**1.0**	**0.4**

Trypsin: Blanks

Table A43. Influence of fat concentration in sample matrix on ultrasonic determination of **trypsin** activity (URT) without BAEE (blanks). Triton X 100 solutions (URT/ HR-US) with different olive oil content, which were used to prepare different Trypsin solutions (URT/ HR-US), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 8 µl of Trypsin solution (URT/ HR-US) to 180 µl of TRIS buffer (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0**	**2.5**	**5**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	0.000017	0.000034	0.000322	0.000199
Std Work 2 ($m \cdot s^{-2}$)	- 0.000009	0.000043	0.000163	0.000165
Mean ($m \cdot s^{-2}$)	**0.000004**	**0.000039**	**0.000243**	**0.000182**
Stdv ($m \cdot s^{-2}$)	0.000019	0.000006	0.000112	0.000024
CV (%)	468.2	16.0	46.3	13.2

Trypsin: Sample solvent without Triton X 100

Table A44. Influence of fat concentration in sample matrix on ultrasonic determination of **trypsin** activity (URT) without emulsifier (Triton X 100). 1 mM Hydrochloric acid solutions (URT/ HR-US) with different olive oil content, which were used to prepare different Trypsin solutions (URT/ HR-US, modified, without Triton X 100), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 8 µl of Trypsin solution (URT/ HR-US, modified, without Triton X 100) to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Olive oil (% (v/v))	**0**	**5**	**7.5**
Std Work 1 ($m \cdot s^{-2}$)	0.000767	0.000748	0.000778
Std Work 2 ($m \cdot s^{-2}$)	0.000789	0.000740	0.000773
Mean ($m \cdot s^{-2}$)	**0.000778**	**0.000744**	**0.000776**
Stdv ($m \cdot s^{-2}$)	0.000015	0.000006	0.000004
CV (%)	**1.97**	**0.75**	**0.46**

Trypsin: Different sample solvents

Table A45. Influence of fat concentration in sample matrix on ultrasonic determination of **trypsin** activity (URT) using different sample solvents in the presence and absence of olive oil. Sample solvents with different olive oil content of 5 % (v/v), which were used to prepare different Trypsin solutions (URT/ HR-US, modified, other sample solvent), were prepared by mixing in a household blender for 10 min. Ultrasonic velocity was measured at 37°C after adding 8 µl of Trypsin solution (URT/ HR-US, modified, other sample solvent) to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

	BB/ NaCl pH 7.5		PP 0.2 M pH 6.8		$CaCl_2$ 0.02 M		$CaCl_2$ 0.02 M/ Triton X 100	
Olive oil (% (v/v))	**0**	**5**	**0**	**5**	**0**	**5**	**0**	**5**
Std Work 1 ($m \cdot s^{-2}$)	0.000720	0.001180	0.000815	0.001021	0.000807	0.000795	0.000781	0.001791
Std Work 2 ($m \cdot s^{-2}$)	0.000721	0.001215	0.000856	-	0.000778	0.000833	0.0008	0.001797
Mean ($m \cdot s^{-2}$)	**0.000720**	**0.001197**	**0.000836**	**0.001021**	**0.000792**	**0.000814**	**0.000791**	**0.001794**
Stdv ($m \cdot s^{-2}$)	0.000000	0.000025	0.000029	-	0.000020	0.000027	0.000013	0.000004
CV (%)	**0.1**	**2.1**	**3.5**	**-**	**2.6**	**3.3**	**1.7**	**0.2**

Appendix A X

Linearity

Table A46. Linearity test of **amylase** activity measurement by ultrasonic method (HR-US). Working standard solutions in Phosphate buffer solution (URT/ HR-US) with different enzyme content were prepared. Ultrasonic velocity was measured at 37°C after adding 20 µl of working standard solution to 1050 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Std Work solution (%)	**0.01**	**0.03**	**0.05**	**0.10**	**0.20**
Std Work 1 ($m \cdot s^{-2}$)	0.000324	0.000535	0.000985	0.001824	0.003229
Std Work 2 ($m \cdot s^{-2}$)	0.000270	0.000541	0.001104	0.001778	0.003131
Mean ($m \cdot s^{-2}$)	**0.000297**	**0.000538**	**0.001044**	**0.001801**	**0.003180**
Stdv ($m \cdot s^{-2}$)	0.000039	0.000005	0.000084	0.000032	0.000070
CV (%)	**13.0**	**0.9**	**8.1**	**1.8**	**2.2**

Table A47. Linearity test of **amylase** activity measurement by ultrasonic method (HR-US FTS). Working standard solutions in Phosphate buffer solution (URT/ HR-US) with different enzyme content were prepared. Ultrasonic velocity was measured at 37°C by using a premix of 15.75 ml Starch solution 3 % (URT/ HR-US) and 300 µl working standard solution. Shown are means of 2 replicates per working standard solution.

Std Work solution (%)	**0.0125**	**0.025**	**0.05**	**0.075**	**0.1**	**0.15**
Std Work 1 ($m \cdot s^{-2}$)	0.00028	0.000517	0.000958	0.001342	0.001656	0.002040
Std Work 2 ($m \cdot s^{-2}$)	0.00029	0.000536	0.000946	0.001342	-	-
Mean ($m \cdot s^{-2}$)	**0.000285**	**0.000527**	**0.000952**	**0.001342**	**0.001656**	**0.002040**
Stdv ($m \cdot s^{-2}$)	0.000007	0.000014	0.000008	0.000000	-	-
CV (%)	**2.5**	**2.6**	**0.9**	**0.0**	**-**	**-**

Table A48. Linearity test of **lipase** activity measurement by ultrasonic method (HR-US). Working standard solutions in Maleate buffer solution pH 7.0 (URT/ HR-US) with different enzyme content were prepared. Ultrasonic velocity was measured at 37°C after adding 20 µl of working standard solution to 1050 µl of Olive oil emulsion (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Std Work solution (%)	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**
Std Work 1 ($m \cdot s^{-2}$)	0.002047	0.004443	0.005787	0.00692	0.008231	0.008754
Std Work 2 ($m \cdot s^{-2}$)	0.00179	0.00455	0.005761	0.007038	0.00827	0.008859
Mean ($m \cdot s^{-2}$)	**0.001918**	**0.004497**	**0.005774**	**0.006979**	**0.008251**	**0.008806**
Stdv ($m \cdot s^{-2}$)	0.000182	0.000076	0.000018	0.000084	0.000028	0.000075
CV (%)	**9.5**	**1.7**	**0.3**	**1.2**	**0.3**	**0.9**

Table A49. Linearity test of **free protease** activity measurement by ultrasonic method (HR-US). Working standard solutions in Borate buffer pH 7.5 (URT/ HR-US) with different enzyme content were prepared. Ultrasonic velocity was measured at 37°C after adding 20 µl of working standard solution to 1050 µl of Casein solution 1.25 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Std Work solution (%)	0.10	0.30	0.50	1.00	1.50
Std Work 1 ($m \cdot s^{-2}$)	0.000194	0.000577	0.000982	0.001897	0.002218
Std Work 2 ($m \cdot s^{-2}$)	0.000160	0.000576	0.000974	0.001826	-
Mean ($m \cdot s^{-2}$)	**0.000177**	**0.000577**	**0.000978**	**0.001862**	**0.002218**
Stdv ($m \cdot s^{-2}$)	0.000024	0.000001	0.000005	0.000050	-
CV (%)	**13.8**	**0.2**	**0.5**	**2.7**	**-**

Table A50. Linearity test of **total protease** activity measurement by ultrasonic method (URT). Drug product I solutions in Calcium chloride solution 0.02 M (URT/ HR-US) with different enzyme content were prepared. Ultrasonic velocity was measured at 37°C using a premix of 1800 µl casein 1.25 % (URT/ HR-US) and 80 µl Drug product solution activated with enterokinase. Shown are means of 2 replicates per working standard solution.

Std Work solution (%)	**0.6**	**1.0**	**1.4**	**1.8**	**2.2**	**2.6**	**3.0**
Std Work 1 ($m \cdot s^{-2}$)	0.000308	0.000528	0.000762	0.000992	0.001220	0.001436	0.001616
Std Work 2 ($m \cdot s^{-2}$)	0.000319	0.000524	0.000757	0.000996	0.001214	0.001423	0.001612
Mean ($m \cdot s^{-2}$)	**0.000313**	**0.000526**	**0.000760**	**0.000994**	**0.001217**	**0.001430**	**0.001614**
Stdv ($m \cdot s^{-2}$)	0.000008	0.000003	0.000003	0.000003	0.000004	0.000009	0.000003
CV (%)	**2.5**	**0.6**	**0.5**	**0.3**	**0.3**	**0.6**	**0.2**

Table A51. Linearity test of **free trypsin** activity measurement by ultrasonic method (URT). Working standard solutions in Triton X 100 solution (URT/ HR-US) with different enzyme content were prepared. Ultrasonic velocity was measured at 37°C after adding 4 µl of working standard solution to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Std Work solution (%)	**0.8**	**0.9**	**1.0**	**1.1**	**1.2**
Std Work 1 ($m \cdot s^{-2}$)	0.000677	0.000757	0.000793	0.000874	0.001024
Std Work 2 ($m \cdot s^{-2}$)	0.000680	-	0.000820	-	0.000973
Std Work 3 ($m \cdot s^{-2}$)	0.000619	-	0.000789	-	0.000959
Mean ($m \cdot s^{-2}$)	**0.000658**	**0.000757**	**0.000800**	**0.000874**	**0.000986**
Stdv ($m \cdot s^{-2}$)	0.000034	-	0.000017	-	0.000034
CV (%)	**5.2**	**-**	**2.1**	**-**	**3.4**

Table A52. Linearity test of **free trypsin** activity measurement by ultrasonic method (URT) using FIP standard. FIP standard solutions in Triton X 100 solution (URT/ HR-US) with different enzyme content were prepared. Ultrasonic velocity was measured at 37°C after adding 4 µl of FIP standard solution to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Std FIP solution (%)	**0.025**	**0.028**	**0.031**	**0.034**	**0.038**
Std Work 1 ($m \cdot s^{-2}$)	0.000686	0.000793	0.000820	0.000923	0.000910
Std Work 2 ($m \cdot s^{-2}$)	0.000697	0.000743	0.000800	0.000890	0.001031
Std Work 3 ($m \cdot s^{-2}$)	-	-	0.000845	-	0.000998
Std Work 4 ($m \cdot s^{-2}$)	-	-	0.000831	-	0.001026
Mean ($m \cdot s^{-2}$)	**0.000691**	**0.000768**	**0.000824**	**0.000906**	**0.001018**
Stdv ($m \cdot s^{-2}$)	0.000007	0.000035	0.000019	0.000024	0.000056
CV (%)	**1.1**	**4.6**	**2.3**	**2.6**	**5.5**

Appendix A XI

Small temperature differences

Table A53. Influence of small temperature differences on ultrasonic determination of **amylase** activity (URT). Ultrasonic velocity was measured at different temperatures after adding 4 µl of Amylase solution (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**36.8**	**36.9**	**37.0**	**37.1**	**37.2**
Std Work 1 ($m \cdot s^{-2}$)	0.001283	0.001317	0.001344	0.001309	0.001333
Std Work 2 ($m \cdot s^{-2}$)	0.001282	0.001274	0.001370	0.001344	0.001280
Mean ($m \cdot s^{-2}$)	0.001282	0.001295	0.001357	0.001326	0.001306
Stdv ($m \cdot s^{-2}$)	0.000001	0.000031	0.000018	0.000025	0.000038
CV (%)	**0.1**	**2.4**	**1.3**	**1.2**	**2.9**

Table A54. Influence of small temperature differences on ultrasonic determination of **amylase** activity (HR-US). Ultrasonic velocity was measured at different temperatures after adding 20 µl of Amylase solution (URT/ HR-US) to 1050 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**36.8**	**36.9**	**37.0**	**37.1**	**37.2**
Std Work 1 ($m \cdot s^{-2}$)	0.001283	0.001317	0.001344	0.001309	0.001333
Std Work 2 ($m \cdot s^{-2}$)	0.001282	0.001274	0.001370	0.001344	0.001280
Mean ($m \cdot s^{-2}$)	0.001282	0.001295	0.001357	0.001326	0.001306
Stdv ($m \cdot s^{-2}$)	0.000001	0.000031	0.000018	0.000025	0.000038
CV (%)	**0.1**	**2.4**	**1.3**	**1.2**	**2.9**

Table A55. Influence of small temperature differences on ultrasonic determination of **lipase** activity (HR-US). Ultrasonic velocity was measured at different temperatures after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**36.8**	**36.9**	**37**	**37.1**	**37.2**
Std Work 1 ($m \cdot s^{-2}$)	0.005937	0.005822	0.005691	0.005732	0.005936
Std Work 2 ($m \cdot s^{-2}$)	0.005940	0.005832	0.005702	0.005682	0.005886
Mean ($m \cdot s^{-2}$)	**0.005938**	**0.005827**	**0.005696**	**0.005707**	**0.005911**
Stdv ($m \cdot s^{-2}$)	0.000002	0.000007	0.000007	0.000035	0.000035
CV (%)	**0.0**	**0.1**	**0.1**	**0.6**	**0.6**

Table A56. Influence of small temperature differences on ultrasonic determination of **free protease** activity (URT). Ultrasonic velocity was measured at different temperatures after adding 4 µl of Free protease solution (URT/ HR-US) to 180 µl of Casein solution 0.625 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**36.8**	**36.9**	**37.0**	**37.1**	**37.2**
Std Work 1 ($m \cdot s^{-2}$)	0.001092	0.001082	0.001106	0.001141	0.001127
Std Work 2 ($m \cdot s^{-2}$)	0.001092	0.001186	0.001114	0.001093	0.001225
Mean ($m \cdot s^{-2}$)	**0.001092**	**0.001134**	**0.001110**	**0.001117**	**0.001176**
Stdv ($m \cdot s^{-2}$)	0.000000	0.000074	0.000006	0.000034	0.000069
CV (%)	**0.0**	**6.5**	**0.5**	**3.0**	**5.9**

Table A57. Influence of small temperature differences on ultrasonic determination of **free protease** activity (HR-US). Ultrasonic velocity was measured at different temperatures after adding 20 µl of Free protease solution (URT/ HR-US) to 1050 µl of Casein solution 1.25 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**36.8**	**36.9**	**37.0**	**37.1**	**37.2**
Std Work 1 ($m \cdot s^{-2}$)	0.000986	0.000993	0.000990	0.001015	0.000999
Std Work 2 ($m \cdot s^{-2}$)	0.000989	0.000975	0.000991	0.000991	0.000991
Mean ($m \cdot s^{-2}$)	**0.000988**	**0.000984**	**0.000990**	**0.001003**	**0.000995**
Stdv ($m \cdot s^{-2}$)	0.000002	0.000013	0.000001	0.000017	0.000006
CV (%)	**0.2**	**1.3**	**0.1**	**1.7**	**0.6**

Table A58. Influence of small temperature differences on ultrasonic determination of **total protease** activity (URT). Ultrasonic velocity was measured at different temperatures using a premix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase. Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**36.8**	**36.9**	**37**	**37.1**	**37.2**
Std Work 1 ($m \cdot s^{-2}$)	0.001002	0.000961	0.000995	0.001023	0.001011
Std Work 2 ($m \cdot s^{-2}$)	0.000991	0.001000	0.001010	0.000990	0.001013
Mean ($m \cdot s^{-2}$)	**0.000996**	**0.000981**	**0.001002**	**0.001006**	**0.001012**
Stdv ($m \cdot s^{-2}$)	0.000008	0.000028	0.000010	0.000023	0.000001
CV (%)	**0.8**	**2.9**	**1.0**	**2.3**	**0.1**

Table A59. Influence of small temperature differences on ultrasonic determination of **total protease** activity (HR-US). Ultrasonic velocity was measured at different temperatures using a premix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase. Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**36.8**	**36.9**	**37**	**37.1**	**37.2**
Std Work 1 ($m \cdot s^{-2}$)	0.001068	0.001058	0.001069	0.001062	0.001083
Std Work 2 ($m \cdot s^{-2}$)	0.001083	0.001039	0.001057	0.001059	0.001076
Mean ($m \cdot s^{-2}$)	**0.001076**	**0.001049**	**0.001063**	**0.001061**	**0.001080**
Stdv ($m \cdot s^{-2}$)	0.000011	0.000013	0.000009	0.000002	0.000004
CV (%)	**1.0**	**1.3**	**0.8**	**0.2**	**0.4**

Table A60. Influence of small temperature differences on ultrasonic determination of **free trypsin** activity (URT). Ultrasonic velocity was measured at different temperatures after adding 8 µl of Trypsin solution (URT/ HR-US) to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**36.8**	**36.9**	**37.0**	**37.1**	**37.2**
Std Work 1 ($m \cdot s^{-2}$)	0.000818	0.000869	0.000813	0.000832	0.000822
Std Work 2 ($m \cdot s^{-2}$)	0.000868	0.000836	0.000807	0.000805	0.000872
Mean ($m \cdot s^{-2}$)	**0.000843**	**0.000852**	**0.000810**	**0.000819**	**0.000847**
Stdv ($m \cdot s^{-2}$)	0.000036	0.000024	0.000004	0.000019	0.000036
CV (%)	**4.2**	**2.8**	**0.6**	**2.4**	**4.2**

Table A61. Influence of small temperature differences on ultrasonic determination of **free trypsin** activity (HR-US). Ultrasonic velocity was measured at different temperatures after adding 20 µl of Trypsin solution (URT/ HR-US) to 1050 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**36.8**	**36.9**	**37**	**37.1**	**37.2**
Std Work 1 ($m \cdot s^{-2}$)	0.000384	0.000379	0.000368	0.000384	0.000391
Std Work 2 ($m \cdot s^{-2}$)	0.000369	0.000377	0.000390	0.000377	0.000393
Mean ($m \cdot s^{-2}$)	**0.000376**	**0.000378**	**0.000379**	**0.000380**	**0.000392**
Stdv ($m \cdot s^{-2}$)	0.000011	0.000001	0.000016	0.000005	0.000002
CV (%)	**2.9**	**0.4**	**4.1**	**1.3**	**0.4**

Appendix A XII

Wide temperature ranges

Table A62. Influence of wide temperature differences on ultrasonic determination of **amylase** activity (URT). Ultrasonic velocity was measured at different temperatures after adding 4 µl of a 0.1 % working standard solution (solvent: Phosphate buffer solution (URT/ HR-US)) to 180 µl of Starch solution 1 % (aqueous). Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**20**	**25**	**30**
Std Work 1 ($m \cdot s^{-2}$)	0.001195	0.001386	0.001212
Std Work 2 ($m \cdot s^{-2}$)	0.001075	0.001309	0.001525
Mean ($m \cdot s^{-2}$)	**0.001135**	**0.001348**	**0.001369**
Stdv ($m \cdot s^{-2}$)	0.000085	0.000054	0.000221
CV (%)	**7.5**	**4.0**	**16.1**

Table A63. Influence of wide temperature differences on ultrasonic determination of **amylase** activity (URT). Ultrasonic velocity was measured at different temperatures after adding 4 µl of Amylase solution (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**25**	**31**	**37**
Std Work 1 ($m \cdot s^{-2}$)	0.000722	0.000998	0.001288
Std Work 2 ($m \cdot s^{-2}$)	0.000692	0.000991	0.001333
Mean ($m \cdot s^{-2}$)	**0.000707**	**0.000995**	**0.001310**
Stdv ($m \cdot s^{-2}$)	0.000021	0.000005	0.000032
CV (%)	**3.0**	**0.5**	**2.4**

Table A64. Influence of wide temperature differences on ultrasonic determination of **free protease** activity (URT). Ultrasonic velocity was measured at different temperatures after adding 4 µl of a 0.5 % working standard solution (aqueous) to 180 µl of Casein solution 0.625 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**27**	**32**	**37**
Std Work 1 ($m \cdot s^{-2}$)	0.000566	0.000755	0.001094
Std Work 2 ($m \cdot s^{-2}$)	0.000553	0.000783	0.001102
Mean ($m \cdot s^{-2}$)	**0.000560**	**0.000769**	**0.001098**
Stdv ($m \cdot s^{-2}$)	0.000009	0.000020	0.000005
CV (%)	**1.6**	**2.6**	**0.5**

Table A65. Influence of wide temperature differences on ultrasonic determination of **total protease** activity (URT). Ultrasonic velocity was measured at different temperatures using a premix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase. Shown are means of 2 replicates per working standard solution.

Temperature (°C)	**27**	**32**	**37**
Std Work 1 ($m \cdot s^{-2}$)	0.000418	0.000653	0.000995
Std Work 2 ($m \cdot s^{-2}$)	0.000413	0.000637	0.001010
Mean ($m \cdot s^{-2}$)	**0.000416**	**0.000645**	**0.001002**
Stdv ($m \cdot s^{-2}$)	0.000004	0.000011	0.000010
CV (%)	**0.9**	**1.7**	**1.0**

Table A66. Influence of wide temperature differences on ultrasonic determination of **free trypsin** activity (URT). Ultrasonic velocity was measured at different temperatures after adding 8 µl of Trypsin solution (URT/ HR-US) to 180 µl of BAEE solution (URT/ HR-US).

Temperature (°C)	**36.8**	**36.9**	**37.0**	**37.1**	**37.2**
Std Work 1 ($m \cdot s^{-2}$)	0.000818	0.000869	0.000813	0.000832	0.000822
Std Work 2 ($m \cdot s^{-2}$)	0.000868	0.000836	0.000807	0.000805	0.000872
Mean ($m \cdot s^{-2}$)	**0.000843**	**0.000852**	**0.000810**	**0.000819**	**0.000847**
Stdv ($m \cdot s^{-2}$)	0.000036	0.000024	0.000004	0.000019	0.000036
CV (%)	**4.2**	**2.8**	**0.6**	**2.4**	**4.2**

Appendix A XIII

Concentration of substrate

Table A67. Influence of substrate concentration on ultrasonic determination of **amylase** activity (HR-US). Ultrasonic velocity was measured after adding 20 µl of Amylase solution (URT/ HR-US) to 1050 µl of substrate solution with varied starch content. Shown are means of 2 replicates per working standard solution.

c Starch (% (w/v))	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Std Work 1 ($m \cdot s^{-2}$)	0.000869	0.001002	0.000991	0.001002	0.001010	0.001072	0.001079	0.001031
Std Work 2 ($m \cdot s^{-2}$)	0.000964	0.001005	0.001017	0.000975	0.001038	0.001053	0.001043	0.001024
Mean ($m \cdot s^{-2}$)	**0.000917**	**0.001003**	**0.001004**	**0.000988**	**0.001024**	**0.001062**	**0.001061**	**0.001028**
Stdv ($m \cdot s^{-2}$)	0.000067	0.000002	0.000019	0.000019	0.000019	0.000014	0.000026	0.000005
CV (%)	**7.4**	**0.2**	**1.9**	**1.9**	**1.9**	**1.3**	**2.5**	**0.5**

Table A68. Influence of substrate concentration on ultrasonic determination of **lipase** activity (HR-US). Ultrasonic velocity was measured after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of substrate emulsion with varied olive oil content. Shown are means of 2 replicates per working standard solution.

c Olive oil in pre-emulsion (% (w/v))	4	6	8	10
c Olive oil in completed emulsion (% (w/v))	**2**	**3**	**4**	**5**
Std Work 1 ($m \cdot s^{-2}$)	0.005665	0.005635	0.005525	0.005616
Std Work 2 ($m \cdot s^{-2}$)	0.005667	0.005624	0.005584	0.005824
Mean ($m \cdot s^{-2}$)	**0.005666**	**0.00563**	**0.005554**	**0.00572**
Stdv ($m \cdot s^{-2}$)	0.000002	0.000007	0.000042	0.000147
CV (%)	**0.0**	**0.1**	**0.8**	**2.6**

Table A69. Influence of substrate concentration on ultrasonic determination of **free protease** activity (HR-US). Ultrasonic velocity was measured after adding 4 µl of Free protease solution (URT/ HR-US) to 180 µl of substrate solution with varied casein content. Shown are means of 2 replicates per working standard solution.

c Casein (% (w/v))	**0.313**	**0.625**	**0.938**	**1.25**
Std Work 1 ($m \cdot s^{-2}$)	0.001085	0.001073	0.000928	0.001127
Std Work 2 ($m \cdot s^{-2}$)	0.001038	0.001059	0.001092	0.001105
Mean ($m \cdot s^{-2}$)	**0.001062**	**0.001066**	**0.001010**	**0.001116**
Stdv ($m \cdot s^{-2}$)	0.000033	0.000009	0.000116	0.000016
CV (%)	**3.1**	**0.9**	**11.5**	**1.4**

Table A70. Influence of substrate concentration on ultrasonic determination of **total protease** activity (URT). Ultrasonic velocity was measured using a premix of 1800 µl substrate solution with varied casein content and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase. Shown are means of 2 replicates per working standard solution.

c Casein (% (w/v))	**0.63**	**1.25**	**1.88**	**2.5**
Std Work 1 ($m \cdot s^{-2}$)	0.001069	0.000988	0.000968	0.000918
Std Work 2 ($m \cdot s^{-2}$)	0.001053	0.001011	0.001010	0.000934
Mean ($m \cdot s^{-2}$)	**0.001061**	**0.000999**	**0.000989**	**0.000926**
Stdv ($m \cdot s^{-2}$)	0.000011	0.000017	0.000029	0.000011
CV (%)	**1.1**	**1.7**	**3.0**	**1.2**

Table A71. Influence of substrate concentration on ultrasonic determination of **free trypsin** activity (URT). Ultrasonic velocity was measured after adding 8 µl of Trypsin solution (URT/ HR-US) to 180 µl of substrate solution with varied BAEE content. Shown are means of 2 replicates per working standard solution.

c BAEE (% (w/v))	**0.1**	**0.2**	**0.3**	**0.4**
c BAEE (mmol·l^{-1})	**2.9**	**5.9**	**8.7**	**11.7**
Std Work 1 (m·s^{-2})	0.000877	0.000852	0.000837	0.000816
Std Work 2 (m·s^{-2})	0.000913	0.000842	0.000817	0.000815
Mean (m·s^{-2})	**0.000895**	**0.000847**	**0.000827**	**0.000816**
Stdv (m·s^{-2})	0.000025	0.000007	0.000014	0.000001
CV (%)	**2.8**	**0.8**	**1.7**	**0.1**

Appendix A XIV

Stability of substrate preparation (and Lipase: Operating time of Microfluidizer)

Table A72. Test of starch preparation stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of **amylase** activity (URT). Ultrasonic velocity was measured after adding 4 µl of Amylase solution (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US) after varied storage times. Shown are means of 2 replicates per working standard solution.

Time (h)	**0**	**24**	**48**
Std Work 1 ($m \cdot s^{-2}$)	0.001190	0.001220	0.001162
Std Work 2 ($m \cdot s^{-2}$)	0.001214	0.001256	0.001140
Mean ($m \cdot s^{-2}$)	**0.001202**	**0.001238**	**0.001151**
Stdv ($m \cdot s^{-2}$)	0.000017	0.000025	0.000015
CV (%)	**1.4**	**2.0**	**1.3**

Table A73. Test of olive oil preparation stability choosing different operating times of the Microfluidizer (MF) before the emulsion was processed evaluated to test applicability to ultrasonic velocity measurements for determination of **lipase** activity (HR-US). The final emulsions were used without storage (0 hours) (**1. Trial**). Ultrasonic velocity was measured after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Operating time of MF before emulsion processing (min)	**0**	**30**	**60**
Std Work 1 ($m \cdot s^{-2}$)	0.005812	0.005723	0.005751
Std Work 2 ($m \cdot s^{-2}$)	0.005665	0.005639	0.005718
Mean ($m \cdot s^{-2}$)	**0.005738**	**0.005681**	**0.005734**
Stdv ($m \cdot s^{-2}$)	0.000104	0.000059	0.000023
CV (%)	**1.8**	**1.0**	**0.4**

Table A74. Test of olive oil preparation stability choosing different operating times of the Microfluidizer (MF) before the emulsion was processed evaluated to test applicability to ultrasonic velocity measurements for determination of **lipase** activity (HR-US). The final emulsions were used without storage (0 hours) (**2. Trial**). Ultrasonic velocity was measured after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Operating time of MF before emulsion processing (min)	**0**	**30**	**60**
Std Work 1 ($m \cdot s^{-2}$)	0.005735	0.005715	0.005666
Std Work 2 ($m \cdot s^{-2}$)	0.005741	0.005702	0.005614
Mean ($m \cdot s^{-2}$)	**0.005738**	**0.005708**	**0.00564**
Stdv ($m \cdot s^{-2}$)	0.000004	0.000009	0.000037
CV (%)	**0.1**	**0.2**	**0.7**

Table A75. Test of olive oil preparation stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of **lipase** activity (HR-US) operating the Microfluidizer **0 min** before the emulsion was processed (**1. Trial)**. Ultrasonic velocity was measured after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US) after storage of 7, 14 and 21 d using the original mixture or a mixture of the emulsion and freshly prepared bile salts solution. Shown are means of 2 replicates per working standard solution.

	Original mixture			**Fresh bile salts solution**		
Time (d)	**7**	**14**	**21**	**7**	**14**	**21**
Std Work 1 ($m \cdot s^{-2}$)	0.005828	0.005695	0.005698	0.005883	0.005815	0.005623
Std Work 2 ($m \cdot s^{-2}$)	0.005667	0.005723	0.005714	0.005893	0.005809	0.005714
Mean ($m \cdot s^{-2}$)	**0.005747**	**0.005709**	**0.005706**	**0.005888**	**0.005812**	**0.005669**
Stdv ($m \cdot s^{-2}$)	0.000114	0.000020	0.000011	0.000007	0.000005	0.000065
CV (%)	**2.0**	**0.4**	**0.2**	**0.1**	**0.1**	**1.1**

Table A76. Test of olive oil preparation stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of **lipase** activity (HR-US) operating the Microfluidizer **30 min** before the emulsion was processed (**1. Trial)**. Ultrasonic velocity was measured after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US) after storage of 7, 14 and 21 d using the original mixture or a mixture of the emulsion and freshly prepared bile salts solution. Shown are means of 2 replicates per working standard solution.

	Original mixture			Fresh bile salts solution		
Time (d)	**7**	**14**	**21**	**7**	**14**	**21**
Std Work 1 ($m \cdot s^{-2}$)	0.005643	0.005618	0.005523	0.005799	0.005727	0.005606
Std Work 2 ($m \cdot s^{-2}$)	0.005661	0.005611	0.005638	0.005878	0.005839	0.005511
Mean ($m \cdot s^{-2}$)	**0.005652**	**0.005614**	**0.005581**	**0.005839**	**0.005783**	**0.005559**
Stdv ($m \cdot s^{-2}$)	0.000013	0.000005	0.000081	0.000056	0.000079	0.000067
CV (%)	**0.2**	**0.1**	**1.5**	**1.0**	**1.4**	**1.2**

Table A77. Test of olive oil preparation stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of **lipase** activity (HR-US) operating the Microfluidizer **60 min** before the emulsion was processed (**1. Trial)**. Ultrasonic velocity was measured after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US) after storage of 7, 14 and 21 d using the original mixture or a mixture of the emulsion and freshly prepared bile salts solution. Shown are means of 2 replicates per working standard solution.

	Original mixture			Fresh bile salts solution		
Time (d)	**7**	**14**	**21**	**7**	**14**	**21**
Std Work 1 ($m \cdot s^{-2}$)	0.005714	0.00558	0.005533	0.005870	0.005672	0.005594
Std Work 2 ($m \cdot s^{-2}$)	0.005781	0.005737	0.005511	0.005793	0.005639	0.005696
Mean ($m \cdot s^{-2}$)	**0.005747**	**0.005659**	**0.005522**	**0.005832**	**0.005655**	**0.005645**
Stdv ($m \cdot s^{-2}$)	0.000048	0.000111	0.000016	0.000055	0.000023	0.000072
CV (%)	**0.8**	**2.0**	**0.3**	**0.9**	**0.4**	**1.3**

Table A78. Test of olive oil preparation stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of **lipase** activity (HR-US) operating the Microfluidizer **0 min** before the emulsion was processed (**2. Trial)**. Ultrasonic velocity was measured after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US) after storage of 7, 14 and 21 d using the original mixture or a mixture of the emulsion and freshly prepared bile salts solution. Shown are means of 2 replicates per working standard solution.

	Original mixture			Fresh bile salts solution		
Time (d)	**7**	**14**	**21**	**7**	**14**	**21**
Std Work 1 ($m \cdot s^{-2}$)	0.005883	0.005844	0.005672	0.005807	0.005769	0.005648
Std Work 2 ($m \cdot s^{-2}$)	0.005868	0.005832	0.005694	0.005742	0.005982	0.005611
Mean ($m \cdot s^{-2}$)	**0.005875**	**0.005838**	**0.005683**	**0.005774**	**0.005875**	**0.00563**
Stdv ($m \cdot s^{-2}$)	0.000010	0.000008	0.000016	0.000046	0.000151	0.000026
CV (%)	**0.2**	**0.2**	**0.3**	**0.8**	**2.6**	**0.5**

Table A79. Test of olive oil preparation stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of **lipase** activity (HR-US) operating the Microfluidizer **30 min** before the emulsion was processed (**2. Trial)**. Ultrasonic velocity was measured after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US) after storage of 7, 14 and 21 d using the original mixture or a mixture of the emulsion and freshly prepared bile salts solution. Shown are means of 2 replicates per working standard solution.

	Original mixture			Fresh bile salts solution		
Time (d)	**7**	**14**	**21**	**7**	**14**	**21**
Std Work 1 ($m \cdot s^{-2}$)	0.005763	0.00578	0.00555	0.005752	0.005545	0.005684
Std Work 2 ($m \cdot s^{-2}$)	0.005756	0.00590	0.005833	0.005666	0.005485	0.005594
Mean ($m \cdot s^{-2}$)	**0.005759**	**0.00584**	**0.005691**	**0.005709**	**0.005515**	**0.005639**
Stdv ($m \cdot s^{-2}$)	0.000005	0.000085	0.000200	0.000061	0.000043	0.000063
CV (%)	**0.1**	**1.5**	**3.5**	**1.1**	**0.8**	**1.1**

Table A80. Test of olive oil preparation stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of **lipase** activity (HR-US) operating the Microfluidizer **60 min** before the emulsion was processed (**2. Trial)**. Ultrasonic velocity was measured after adding 20 µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US) after storage of 7, 14 and 21 d using the original mixture or a mixture of the emulsion and freshly prepared bile salts solution. Shown are means of 2 replicates per working standard solution.

	Original mixture			Fresh bile salts solution		
Time (d)	**7**	**14**	**21**	**7**	**14**	**21**
Std Work 1 ($m \cdot s^{-2}$)	0.005701	0.005704	0.005549	0.00564	0.005686	0.005584
Std Work 2 ($m \cdot s^{-2}$)	0.005615	0.005621	0.005839	0.005707	0.00543	0.005672
Mean ($m \cdot s^{-2}$)	**0.005658**	**0.005662**	**0.005694**	**0.005673**	**0.005558**	**0.005628**
Stdv ($m \cdot s^{-2}$)	0.000061	0.000059	0.000205	0.000047	0.000181	0.000062
CV (%)	**1.1**	**1.0**	**3.6**	**0.8**	**3.3**	**1.1**

Table A81. Test of casein preparation stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of **free protease** activity (URT). Ultrasonic velocity was measured after adding 4 µl of 0.5 % standard working solution (aqueous) to 180 µl of Casein solution 0.625 % (URT/ HR-US) after varied storage times. Shown are means of 2 replicates per working standard solution.

Time (d)	**0**	**24**	**48**
Std Work 1 ($m \cdot s^{-2}$)	0.001223	0.001218	0.001132
Std Work 2 ($m \cdot s^{-2}$)	0.001182	0.001181	0.001364
Mean ($m \cdot s^{-2}$)	**0.001203**	**0.001199**	**0.001248**
Stdv ($m \cdot s^{-2}$)	0.000029	0.000026	0.000164
CV (%)	**2.4**	**2.2**	**13.1**

Table A82. Test of casein preparation stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of **total protease** activity (URT). Ultrasonic velocity was measured using a premix of 1800 µl Casein solution 1.25 % (URT/ HR-US) after varied storage times and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase. Shown are means of 2 replicates per working standard solution.

Time (d)	**0**	**24**	**48**	**72**	**96**
Std Work 1 ($m \cdot s^{-2}$)	0.000995	0.000988	0.000995	0.001000	0.000966
Std Work 2 ($m \cdot s^{-2}$)	0.001010	0.001002	0.000992	0.000992	0.000984
Mean ($m \cdot s^{-2}$)	**0.001002**	**0.000995**	**0.000993**	**0.000996**	**0.000975**
Stdv ($m \cdot s^{-2}$)	0.000010	0.000010	0.000002	0.000006	0.000012
CV (%)	**1.0**	**1.0**	**0.2**	**0.6**	**1.3**

Table A83. Test of BAEE preparation stability over time evaluated to test applicability to ultrasonic velocity measurements for determination of **free trypsin** activity (URT). Ultrasonic velocity was measured after adding 8 µl of Trypsin solution (URT/ HR-US) to 180 µl of substrate solution after varied storage times. Shown are means of 2 replicates per working standard solution.

Time (d)	**0**	**24**	**48**
Std Work 1 ($m \cdot s^{-2}$)	0.000852	0.000824	0.000828
Std Work 2 ($m \cdot s^{-2}$)	0.000842	0.000836	0.000823
Mean ($m \cdot s^{-2}$)	**0.000847**	**0.000830**	**0.000826**
Stdv ($m \cdot s^{-2}$)	0.000007	0.000009	0.000004
CV (%)	**0.8**	**1.0**	**0.5**

Appendix A XV

Extraction (Dissolving) time

Table A84. Influence of Extraction (Dissolving) time of pancreas powder in sample solvent on ultrasonic determination of **amylase** activity (URT). Amylase solutions (URT/ HR-US) were prepared dissolving working standard for different times in Phosphate buffer solution (URT/ HR-US). Ultrasonic velocity was measured at 37°C after adding 4µl of Amylase solution (URT/ HR-US) to 180 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Extraction (Dissolving) time (min)	Std Work 1 ($m \cdot s^{-2}$)	Std Work 2 ($m \cdot s^{-2}$)
10	0.001206	0.001233
25	0.001253	0.001171
40	0.001359	0.001193
55	0.001269	0.001300
70	0.001250	0.001293
85	0.001237	0.001362
Mean ($m \cdot s^{-2}$)	**0.001263**	**0.001259**
Stdv ($m \cdot s^{-2}$)	0.000052	0.000072
CV (%)	**4.1**	**5.7**

Table A85. Influence of Extraction (Dissolving) time of pancreas powder in sample solvent on ultrasonic determination of **lipase** activity (HR-US). Lipase solutions (URT/ HR-US) were prepared dissolving working standard for different times in Maleate buffer solution pH 7.0 (URT/ HR-US). Ultrasonic velocity was measured at 37°C after adding 20µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Extraction (Dissolving) time (min)	Std Work 1 ($m \cdot s^{-2}$)	Std Work 2 ($m \cdot s^{-2}$)	Std Work 3 ($m \cdot s^{-2}$)	Std Work 4 ($m \cdot s^{-2}$)
10	0.005805	0.005641	0.005797	0.005822
25	0.005741	0.005733	0.005811	0.00583
40	0.005789	0.005615	0.005883	0.005796
55	0.005597	0.005638	0.005861	0.005817
70	0.005579	0.005616	0.005693	0.005755
85	0.005648	0.005745	0.00573	0.005785
100	0.005342	0.005663	0.005811	0.005624
Mean ($m \cdot s^{-2}$)	**0.005643**	**0.005664**	**0.005798**	**0.005776**
Stdv ($m \cdot s^{-2}$)	0.000160	0.000054	0.000067	0.000072
CV (%)	**2.8**	**1.0**	**1.2**	**1.2**

Table A86. Influence of Extraction (Dissolving) time of pancreas powder in sample solvent on ultrasonic determination of **free protease** activity (URT). Free protease solutions (URT/ HR-US) were prepared dissolving working standard for different times in Borate buffer pH 7.5 (URT/ HR-US). Ultrasonic velocity was measured at 37°C using a premix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 40 µl Free protease solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Extraction (Dissolving) time (min)	Std Work 1 ($m \cdot s^{-2}$)	Std Work 2 ($m \cdot s^{-2}$)
10	0.001068	0.001013
25	0.001055	0.001077
40	0.001087	0.001014
55	0.001086	0.001043
70	0.001048	0.000999
85	0.001035	0.001030
Mean ($m \cdot s^{-2}$)	**0.001063**	**0.001029**
Stdv ($m \cdot s^{-2}$)	0.000021	0.000028
CV (%)	**2.0**	**2.7**

Table A87. Influence of Extraction (Dissolving) time of pancreas powder in sample solvent on ultrasonic determination of **total protease** activity (URT). Total protease solutions (URT/ HR-US) were prepared dissolving working standard for different times in Calcium chloride solution 0.02 M (URT/ HR-US) and subsequently activating with enterokinase . Ultrasonic velocity was measured at 37°C using a premix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

Extraction (Dissolving) time (min)	Std Work 1 ($m \cdot s^{-2}$)	Std Work 2 ($m \cdot s^{-2}$)
10	0.000988	0.001011
25	0.000988	0.000999
40	0.000967	0.001003
55	0.000989	0.000975
70	0.000973	0.000971
85	0.000968	0.000972
Mean ($m \cdot s^{-2}$)	**0.000979**	**0.000988**
Stdv ($m \cdot s^{-2}$)	0.000011	0.000018
CV (%)	**1.1**	**1.8**

Table A88. Influence of Extraction (Dissolving) time of pancreas powder in sample solvent on ultrasonic determination of **free trypsin** activity (URT). Trypsin solutions (URT/ HR-US) were prepared dissolving working standard for different times in Triton X 100 solution (URT/ HR-US). Ultrasonic velocity was measured at 37°C after adding 8 µl of Trypsin solution (URT/ HR-US) to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 replicates per working standard solution. Shown are means of 2 replicates per working standard solution.

Extraction (Dissolving) time (min)	Std Work 1 ($m \cdot s^{-2}$)	Std Work 2 ($m \cdot s^{-2}$)
10	0.000793	0.000804
25	0.000826	0.000812
40	0.000825	0.000810
55	0.000787	0.000818
70	0.000833	0.000841
85	0.000848	0.000880
Mean ($m \cdot s^{-2}$)	**0.000819**	**0.000827**
Stdv ($m \cdot s^{-2}$)	0.000024	0.000028
CV (%)	**2.9**	**3.4**

Appendix A XVI

Lipase: Potential influencing factors of lipase activity depending on substrate emulsion

Table A89. Influence of bile salt concentration in olive oil emulsion on ultrasonic determination of **lipase** activity (HR-US) **(1. part)**. Ultrasonic velocity was measured at 37°C after adding 20µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US) with varied bile salt content. Shown are means of 2 replicates per working standard solution.

c Bile salts in Bile salts solution (% (w/v))	0.21	0.42	0.63	0.84	1.26
c Bile salts in olive oil emulsion (% (w/v))	**0.11**	**0.21**	**0.32**	**0.42**	**0.63**
Std Work 1 ($m \cdot s^{-2}$)	0.000019	0.000115	0.001657	0.005823	0.005908
Std Work 2 ($m \cdot s^{-2}$)	0.000004	0.000138	0.001511	0.005757	0.005915
Mean ($m \cdot s^{-2}$)	**0.000012**	**0.000126**	**0.001584**	**0.005790**	**0.005911**
Stdv ($m \cdot s^{-2}$)	0.000010	0.000016	0.000103	0.000047	0.000004
CV (%)	**88.4**	**12.7**	**6.5**	**0.8**	**0.1**

Table A90. Influence of bile salt concentration in olive oil emulsion on ultrasonic determination of **lipase** activity (HR-US) **(2. part)**. Ultrasonic velocity was measured at 37°C after adding 20µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US) with varied bile salt content. Shown are means of 2 replicates per working standard solution.

c Bile salts in Bile salts solution (% (w/v))	0.84	1.26	1.68	2.10	0.21
c Bile salts in olive oil emulsion (% (w/v))	**0.42**	**0.63**	**0.84**	**1.05**	**0.11**
Std Work 1 ($m \cdot s^{-2}$)	0.005867	0.005701	0.005519	0.005220	0.000024
Std Work 2 ($m \cdot s^{-2}$)	0.005728	0.005733	0.005382	-	-
Mean ($m \cdot s^{-2}$)	**0.005798**	**0.005717**	**0.005451**	**0.005220**	**0.000024**
Stdv ($m \cdot s^{-2}$)	0.000098	0.000022	0.000097	-	-
CV (%)	**1.7**	**0.4**	**1.8**	**-**	**-**

Table A91. Influence of olive oil emulsion pH on ultrasonic determination of **lipase** activity (HR-US). Ultrasonic velocity was measured at 37°C after adding 20µl of Lipase solution (URT/ HR-US) to 1050 µl of Olive oil emulsion (URT/ HR-US) with varied pH. Shown are means of 2 replicates per working standard solution.

pH of emulsion	**7.2**	**7.8**	**8.4**	**9**	**9.5**
Std Work 1 ($m \cdot s^{-2}$)	0.005710	0.007849	0.008419	0.008118	0.007509
Std Work 2 ($m \cdot s^{-2}$)	0.005700	0.008005	0.008786	0.007753	0.007710
Mean ($m \cdot s^{-2}$)	**0.005705**	**0.007927**	**0.008603**	**0.007936**	**0.007610**
Stdv ($m \cdot s^{-2}$)	0.000007	0.000110	0.000260	0.000258	0.000143
CV (%)	**0.1**	**1.4**	**3.0**	**3.3**	**1.9**

Appendix A XVII

Total protease: Activation parameters

Table A92. Influence of enterokinase concentration on ultrasonic determination of **total protease** activity (URT). Ultrasonic velocity was measured at 37°C using a premix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated by using different concentrations of enterokinase solution. Shown are means of 2 replicates per working standard solution.

c Enterokinase (% (w/v))	**0.85**	**1.25**	**1.65**	**1.65**	**2.05**	**2.5**
Std Work 1 ($m \cdot s^{-2}$)	0.000975	0.001040	0.001022	0.001009	0.001004	0.001065
Std Work 2 ($m \cdot s^{-2}$)	0.000933	0.000990	0.000999	0.000993	0.000994	0.001087
Mean ($m \cdot s^{-2}$)	**0.000954**	**0.001015**	**0.001010**	**0.001001**	**0.000999**	**0.001076**
Stdv ($m \cdot s^{-2}$)	0.000030	0.000035	0.000017	0.000011	0.000007	0.000016
CV (%)	**3.10**	**3.47**	**1.66**	**1.09**	**0.73**	**1.47**

Table A93. Blank test evaluated to test influence of enterokinase concentration on ultrasonic determination of **total protease** activity (URT). Ultrasonic velocity was measured at 37°C using a premix of casein solution 1.25 % and $CaCl_2$ solution (URT/ HR-US) "activated" by using different concentrations of enterokinase solution. Shown are means of 2 replicates per working standard solution.

c Enterokinase (% (w/v))	**0.85**	**1.25**	**1.65**	**1.65**	**2.05**	**2.5**
Std Work 1 ($m \cdot s^{-2}$)	0.000030	0.000035	0.000035	0.000069	0.000063	0.000107
Std Work 2 ($m \cdot s^{-2}$)	0.000023	0.000037	-	0.000026	0.000074	0.000093
Mean ($m \cdot s^{-2}$)	**0.000026**	**0.000036**	**0.000035**	**0.000047**	**0.000068**	**0.000100**
Stdv ($m \cdot s^{-2}$)	0.000005	0.000001	-	0.000030	0.000007	0.000009
CV (%)	**17.97**	**3.33**	**-**	**63.26**	**10.96**	**9.44**

Table A94. Influence of storage time of enterokinase solution on ultrasonic determination of **total protease** activity (URT). Ultrasonic velocity was measured at 37°C using a premix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase solution after varied storage times. Shown are means of 2 replicates per working standard solution.

Time (h)	**0**	**24**	**48**	**72**
Std Work 1 ($m \cdot s^{-2}$)	0.000962	0.000998	0.001014	0.001024
Std Work 2 ($m \cdot s^{-2}$)	0.001001	0.000975	0.000991	0.001004
Mean ($m \cdot s^{-2}$)	**0.000982**	**0.000986**	**0.001003**	**0.001014**
Stdv ($m \cdot s^{-2}$)	0.000027	0.000016	0.000016	0.000015
CV (%)	**2.79**	**1.60**	**1.64**	**1.44**

Table A95. Influence of activation time on ultrasonic determination of **total protease** activity (URT). Ultrasonic velocity was measured at 37°C using a premix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase for different times. Shown are means of 2 replicates per working standard solution.

Activation time (min)	0	5	10	15	20
Std Work 1 ($m \cdot s^{-2}$)	0.000959	0.001082	0.001053	0.001075	0.001055
Std Work 2 ($m \cdot s^{-2}$)	0.000918	0.001059	0.001038	0.001029	0.001071
Mean ($m \cdot s^{-2}$)	**0.000939**	**0.001071**	**0.001046**	**0.001052**	**0.001063**
Stdv ($m \cdot s^{-2}$)	0.000029	0.000016	0.000011	0.000033	0.000011
CV (%)	**3.08**	**1.50**	**1.04**	**3.10**	**1.03**

Table A96. Influence of activation time on ultrasonic determination of **total protease** activity (URT). Ultrasonic velocity was measured at 37°C using a premix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US) activated with enterokinase at varied temperatures. Shown are means of 2 replicates per working standard solution.

Activation temperature (°C)	**25**	**30**	**35**	**40**	**45**
Std Work 1 ($m \cdot s^{-2}$)	0.001001	0.000974	0.000961	0.001001	0.001011
Std Work 2 ($m \cdot s^{-2}$)	0.000952	0.000959	0.000983	0.001098	0.001021
Mean ($m \cdot s^{-2}$)	**0.000977**	**0.000966**	**0.000972**	**0.001050**	**0.001016**
Stdv ($m \cdot s^{-2}$)	0.000034	0.000011	0.000015	0.000068	0.000007
CV (%)	**3.50**	**1.10**	**1.58**	**6.51**	**0.70**

Appendix A XVIII

Holding time in dosing system (titration equipment)

Table A97. Influence of holding time in dosing system on ultrasonic determination of **amylase** activity (HR-US) (periodic injection). Ultrasonic velocity was measured at 37°C periodically injecting 4 µl of Amylase solution (URT/ HR-US) via dosing system through a septum into the measurement cell containing 1050 µl Starch solution 6 % (URT/ HR-US).

Volume of Std Work solution periodically added to system (µl)	Total volume of Std Work solution added to system (µl)	Concentration of Std Work (total) in system (µg)	Change of ultrasonic velocity over time ($m \cdot s^{-2}$)	
			Std Work 1	Std Work 2
0	0	0	0	0
4	4	0.001898	0.000203	0.000203
4	8	0.003781	0.000355	0.000410
4	12	0.005650	0.000427	0.000597
4	16	0.007505	0.000524	0.000683
4	20	0.009346	0.000583	0.000757
4	24	0.011173	0.000597	-
4	28	0.012987	0.000631	-
4	32	0.014787	0.000565	-
4	36	0.016575	0.000554	-

Table A98. Influence of holding time in dosing system on ultrasonic determination of **amylase** activity (HR-US) (no periodic injection). Ultrasonic velocity was measured at 37°C directly injecting 20 µl/ 30 µl of Amylase solution (URT/ HR-US) through a septum into the measurement cell containing 1050 µl Starch solution 6 % (URT/ HR-US).

Holding time	Total volume of Std Work solution added to system (µl)	Concentration of Std Work (total) in system (µg)	Change of ultrasonic velocity over time ($m \cdot s^{-2}$)	
			Std Work 1	Std Work 2
short	20	0.009346	0.000982	-
short	30	0.013889	0.001479	-
long	20	0.009346	-	0.000061

Table A99. Influence of holding time in dosing system on ultrasonic determination of **free protease** activity (HR-US) (periodic injection). Ultrasonic velocity was measured at 37°C periodically injecting 4 µl of Free protease solution (URT/ HR-US) via dosing system through a septum into the measurement cell containing 1050 µl Casein 1.25 % (URT/ HR-US).

Volume of Std Work solution periodically added to system (µl)	Total volume of Std Work solution added to system (µl)	Concentration of Std Work (total) in system (µg)	Change of ultrasonic velocity over time ($m \cdot s^{-2}$)	
			Std Work 1	Std Work 2
0	0	0	0	0
4	4	0.001898	0.000203	0.000203
4	8	0.003781	0.000355	0.000410
4	12	0.005650	0.000427	0.000597
4	16	0.007505	0.000524	0.000683
4	20	0.009346	0.000583	0.000757
4	24	0.011173	0.000597	-
4	28	0.012987	0.000631	-
4	32	0.014787	0.000565	-
4	36	0.016575	0.000554	-

Table A100. Influence of holding time in dosing system on ultrasonic determination of **free protease** activity (HR-US) (no periodic injection). Ultrasonic velocity was measured at 37°C directly injecting 20 µl/ 40 µl of Free protease solution (URT/ HR-US) through a septum into the measurement cell containing 1050 µl Casein solution 1.25 % (URT/ HR-US).

Holding time	Total volume of Std Work solution added to system (µl)	Concentration of Std Work (total) in system (µg)	Change of ultrasonic velocity over time ($m \cdot s^{-2}$)
			Std Work 1
short	20	0.009346	0.000924
short	40	0.183486	0.002091

Appendix A XIX

Amylase: Dextrins as alternative substrates

Table A101. Suitability test of **Dextrin 5** as alternative substrate for **amylase** activity measurement by ultrasonic method (URT). Ultrasonic velocity was measured after adding 4 µl of Amylase solution (URT/ HR-US) to 180 µl of substrate solution with varied Dextrin 5 content prepared in Phosphate buffer (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

c Dextrin 5 (substrate) (% (m/v))	**5**	**10**	**20**
Std Work 1 ($m \cdot s^{-2}$)	0.001237	0.001108	-
Std Work 2 ($m \cdot s^{-2}$)	0.001210	0.001127	-
Mean ($m \cdot s^{-2}$)	**0.001223**	**0.001117**	**-**
Stdv ($m \cdot s^{-2}$)	0.000019	0.000013	-
CV (%)	**1.59**	**1.20**	**-**

Table A102. Suitability test of **Dextrin 10** as alternative substrate for **amylase** activity measurement by ultrasonic method (URT). Ultrasonic velocity was measured after adding 4 µl of Amylase solution (URT/ HR-US) to 180 µl of substrate solution with varied Dextrin 10 content prepared in Phosphate buffer (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

c Dextrin 10 (substrate) (% (m/v))	**5**	**10**	**20**
Std Work 1 ($m \cdot s^{-2}$)	0.001095	0.000911	0.000710
Std Work 2 ($m \cdot s^{-2}$)	0.000974	0.000913	0.000800
Mean ($m \cdot s^{-2}$)	**0.001035**	**0.000912**	**0.000755**
Stdv ($m \cdot s^{-2}$)	0.000086	0.000001	0.000064
CV (%)	**8.33**	**0.16**	**8.43**

Table A103. Suitability test of **Dextrin 20** as alternative substrate for **amylase** activity measurement by ultrasonic method (URT). Ultrasonic velocity was measured after adding 4 µl of Amylase solution (URT/ HR-US) to 180 µl of substrate solution with varied Dextrin 20 content prepared in Phosphate buffer (URT/ HR-US). Shown are means of 2 replicates per working standard solution.

c Dextrin 20 (substrate) (% (m/v))	**5**	**10**	**20**
Std Work 1 ($m \cdot s^{-2}$)	0.000651	0.000530	0.000371
Std Work 2 ($m \cdot s^{-2}$)	0.000628	0.000493	0.000354
Mean ($m \cdot s^{-2}$)	**0.000639**	**0.000512**	**0.000362**
Stdv ($m \cdot s^{-2}$)	0.000016	0.000026	0.000012
CV (%)	**2.57**	**5.17**	**3.28**

Appendix A XX

Proteolysis-like material

Table A104. Relative **amylase** activities (Ph.-Eur.-u/ g) in three batches of **proteolysis-like material** with four samples each determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C after adding 4 µl of Amylase solution (URT/ HR-US) (sample or working standard) to 180 µl of Starch solution 6 % (URT/ HR-US). Shown are means of 2 sample solutions with 1 replicate each.

	Batch A			Batch B			Batch C		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)
1	**8569**	169	2.0	**6825**	256	3.8	**7522**	100	1.3
2	**7712**	198	2.6	**6450**	436	6.8	**7213**	446	6.2
3	**7195**	378	5.3	**6525**	383	5.9	**6539**	317	4.9
4	**6964**	359	5.2	**6241**	444	7.1	**6446**	912	14.2

Table A105. Relative **free protease** activities (Ph.-Eur.-u/ g) in three batches of **proteolysis-like material** with four samples each determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C after adding 4 µl of Free protease solution (URT/ HR-US) (sample or working standard) to 180 µl of Casein solution 1.25 % (URT/ HR-US). Shown are means of 2 sample solutions with 1 replicate each.

	Batch A			Batch B			Batch C		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)
1	**438**	15	3.5	**180**	7	3.6	**212**	8	3.6
2	**526**	25	4.7	**576**	39	6.8	**575**	9	1.5
3	**532**	33	6.2	**573**	14	2.4	**531**	8	1.6
4	**537**	15	2.8	**595**	38	6.4	**493**	11	2.3

Table A106. Relative **free protease** activities (Ph.-Eur.-u/ g) in three batches of **proteolysis-like material** with four samples each determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C after adding 4 µl of Trypsin solution (URT/ HR-US) (sample or working standard) to 180 µl of BAEE solution (URT/ HR-US). Shown are means of 2 sample solutions with 1 replicate each.

	Batch A			Batch B			Batch C		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)
1	**22** [29.42 %]	3	13.4	**14** [18.07 %]	1	10.0	**20** [2.86 %]	7	33.5
2	**436** [4.18 %]	7	1.6	**423** [0.48 %]	51	12.2	**412** [8.91 %]	12	3.0
3	**455** [6.29 %]	6	1.4	**389** [1.92 %]	13	3.4	**392** [14.77 %]	17	4.3
4	**400** [5.08 %]	34	8.4	**382** [5.82 %]	24	6.2	**403** [5.40 %]	6	1.6

Appendix A XXI

Proteolysis material I

Table A107. Relative amylase, free protease and free trypsin activities (Ph.-Eur.-u/ g) in **proteolysis I material (Batch I)** determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C after adding Amylase/ Free protease/ Trypsin solution (URT/ HR-US) (sample or working standard) to 180 µl of the respective substrate solution (URT/ HR-US). Shown are means of 2 sample solutions with 1 replicate each.

Time of proteolysis (h)		**0**	**1**	**2**	**3**	**4**	**5**
Amylase	**Mean (u/ g)**	**5,642**	**10,249**	**10,647**	**9,935**	**11,031**	**10,436**
[fresh samples]	Stdev (u/ g)	3,522	328	81	694	309	358
	CV (%)	62.4	3.2	0.8	7.0	2.8	3.4
Protease (free)	**Mean (u/ g)**	**115**	**208**	**219**	**248**	**280**	-
[fresh samples]	Stdev (u/ g)	5	12	6	4	12	-
	CV (%)	4.1	5.8	2.9	1.8	4.1	-
Trypsin (free)	**Mean (u/ ml)**	**14**	**21**	**23**	**31**	**28**	-
[fresh samples]	Stdev (u/ ml)	3	2	4	9	-	-
	CV (%)	18.6	7.4	16.2	28.6	-	-

Table A108. Relative amylase, free protease and free trypsin activities (Ph.-Eur.-u/ g) in **proteolysis I material (Batch II)** determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C after adding Amylase/ Free protease/ Trypsin solution (URT/ HR-US) (sample or working standard) to 180 µl of the respective substrate solution (URT/ HR-US). Shown are means of 2 sample solutions with 1 replicate each.

Time of proteolysis (h)		**0**	**1**	**2**	**3**	**4**	**5**	**5.5**
Amylase	**Mean (u/ g)**	**9,065**	**9,901**	**9,074**	**8,987**	**8,549**	**8,390**	**8,643**
[frozen samples]	Stdev (u/ g)	686	414	676	7	56	420	86
	CV (%)	7.6	4.2	7.5	0.1	0.7	5.0	1.0
Protease (free)	**Mean (u/ g)**	**240**	**246**	**258**	**252**	**312**	**334**	**354**
[frozen samples]	Stdev (u/ g)	10	11	13	13	32	0	0
	CV (%)	4.1	4.5	5.1	5.2	10.3	0.1	0.0
Trypsin (free)	**Mean (u/ ml)**	**47**	**63**	**79**	**100**	**123**	**164**	**189**
[frozen samples]	Stdev (u/ ml)	6	1	10	4	27	6	4
	CV (%)	12.9	1.8	12.4	3.7	22.2	3.7	2.0

Table A109. Relative amylase, free protease and free trypsin activities (Ph.-Eur.-u/ g) in **proteolysis I material (Batch III)** determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C after adding Amylase/ Free protease/ Trypsin solution (URT/ HR-US) (sample or working standard) to 180 µl of the respective substrate solution (URT/ HR-US). Shown are means of 2 sample solutions with 1 replicate each.

Time of proteolysis (h)		**0**	**1**	**2**	**3**	**3.5**
Amylase	**Mean (u/ g)**	**6,433**	**6,684**	**6,327**	**5,866**	**5,968**
[fresh samples]	Stdev (u/ g)	98	764	377	122	772
	CV (%)	1.5	11.4	6.0	2.1	12.9
Protease (free)	**Mean (u/ g)**	**138**	**198**	**195**	**209**	**211**
[frozen samples]	Stdev (u/ g)	15	20	0	13	4
	CV (%)	10.7	10.1	0.0	6.2	2.0
Trypsin (free)	**Mean (u/ ml)**	**26**	**42**	**45**	**53**	**64**
[frozen samples]	Stdev (u/ ml)	1	3	9	0	3
	CV (%)	4.2	7.5	20.4	0.9	4.0

Appendix A XXII

Proteolysis material II

Table A110. Relative **amylase** activities (Ph.-Eur.-u/ g) in three batches of **proteolysis II material** determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C using a pre-mix of 1800 µl Starch solution 3 % (URT/ HR-US) and 40 µl Amylase solution (URT/ HR-US). Shown are means of 2 sample solutions with 2 replicates each.

Time (h)	Batch a (frozen)			Batch b (fresh)			Batch c (frozen)		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)
0	9,629	134	1.4	10,090	44	0.4	9,727	572	5.9
1	9,768	122	1.2	9,523	45	0.5	10,090	97	1.0
2	10,008	262	2.6	9,897	1	0.0	9,909	174	1.8
3	9,806	161	1.6	9,840	142	1.4	10,078	223	2.2
4	9,982	355	3.6	9,662	214	2.2	9,763	441	4.5
5	10,046	61	0.6	9,998	232	2.3	9,872	31	0.3
6	9,997	166	1.7	10,059	64	0.6	9,435	162	1.7
7	9,953	114	1.2	9,970	46	0.5	9,900	55	0.6
8	9,765	86	0.9	9,902	119	1.2	9,698	88	0.9
9	-	-	-	-	-	-	9,535	210	2.2

Table A111. Relative **free protease** activities (Ph.-Eur.-u/ g) in three batches of **proteolysis II material** determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C using a pre-mix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 40 µl Free protease solution (URT/ HR-US) . Shown are means of 2 sample solutions with 2 replicates each.

Time (h)	Batch a (frozen)			Batch b (fresh)			Batch b (frozen)		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)
0	167	14	8.5	179	5	3.1	159	22	13.9
1	240	8	3.4	257	1	0.5	233	19	8.2
2	269	4	1.7	275	1	0.5	266	3	1.2
3	282	2	0.6	305	0	0.2	285	3	0.9
4	293	2	0.6	313	7	2.4	309	3	1.1
5	310	6	1.9	338	6	1.9	310	0	0.1
6	310	10	3.1	335	2	0.5	315	9	3.0
7	322	1	0.2	351	14	3.9	318	1	0.2
8	323	15	4.6	344	8	2.4	327	7	2.1
9	-	-	-	-	-	-	327	1	0.3

Table A112. Relative **total protease** activities (Ph.-Eur.-u/ g) in three batches of **proteolysis II material** determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C using a pre-mix of 1800 µl Casein solution 1.25 % (URT/ HR-US) and 80 µl Total protease solution (URT/ HR-US). Shown are means of 2 sample solutions with 2 replicates each.

Time (h)	Batch a (frozen)			Batch b (fresh)			Batch b (frozen)		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)
0	816	116	14.2	753	16	2.2	802	13	1.6
1	799	73	9.1	744	12	1.7	741	2	0.3
2	714	7	1.0	717	12	1.7	695	12	1.7
3	647	47	7.3	657	1	0.2	635	18	2.8
4	623	8	1.2	633	9	1.5	604	2	0.3
5	555	1	0.2	582	7	1.1	548	4	0.8
6	523	19	3.6	556	7	1.3	512	7	1.4
7	479	7	1.5	516	16	3.1	472	4	0.7
8	406	76	18.6	479	16	3.4	423	36	8.5
9	-	-	-	-	-	-	407	11	2.7

Table A113. Relative **lipase** activities (Ph.-Eur.-u/ g) in three batches of **proteolysis II material** determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C using a pre-mix of 1800 µl Olive oil emulsion (URT/ HR-US) and 33 µl Lipase solution (URT/ HR-US). Shown are means of 2 sample solutions with 2 replicates each.

Time (h)	Batch a (frozen)			Batch b (fresh)			Batch b (frozen)		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)
0	10,015	358	3.6	10,089	63	0.6	9,871	261	2.6
1	9,656	168	1.7	10,041	541	5.4	9,785	746	7.6
2	10,274	39	0.4	10,264	219	2.1	10,086	35	0.4
3	10,193	33	0.3	10,275	262	2.6	9,989	136	1.4
4	10,361	93	0.9	10,658	188	1.8	9,854	70	0.7
5	10,569	186	1.8	10,587	170	1.6	9,432	870	9.2
6	10,212	40	0.4	10,038	101	1.0	9,798	330	3.4
7	10,595	402	3.8	10,237	271	2.7	9,789	496	5.1
8	10,009	254	2.5	10,214	56	0.6	10,230	326	3.2
9	-	-	-	-	-	-	9,692	350	3.6

Table A114. Relative **free trypsin** activities (Ph.-Eur.-u/ g) in three batches of **proteolysis II material** determined by ultrasonic method (URT). Ultrasonic velocity was measured at 37°C using a pre-mix of 1800 µl BAEE solution (URT/ HR-US) and 80 µl Free trypsin solution (URT/ HR-US) . Shown are means of 2 sample solutions with 2 replicates each.

Time (h)	Batch a (frozen)			Batch b (fresh)			Batch b (frozen)		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)
0	18	0.2	1.3	26	1.9	7.3	24	0.1	0.5
1	20	1.5	7.7	40	0.4	1.0	27	0.2	0.8
2	26	1.2	4.5	52	2.2	4.3	27	3.0	11.2
3	37	1.2	3.2	90	11.2	12.5	31	2.8	9.0
4	47	1.0	2.2	93	6.1	6.6	68	3.3	4.9
5	54	2.0	3.7	71	2.2	3.1	60	3.1	5.1
6	62	5.9	9.6	70	4.6	6.7	65	7.0	10.8
7	59	3.1	5.3	72	7.2	9.9	61	0.9	1.5
8	64	1.1	1.8	71	1.5	2.1	50	6.2	12.6
9	-	-	-	-	-	-	57	2.3	4.0

Table A115. Relative **free trypsin** activities (Ph.-Eur.-u/ g) in three batches of **proteolysis II material** determined by Pharmacopoeia method (Ph.Eur.). Ultrasonic velocity was measured at 25°C and pH 8 using 0.02 M NaOH as titration solution. Shown are means of 2 sample solutions with 2 replicates each.

Time (h)	Batch a (frozen)			Batch b (fresh)			Batch b (frozen)		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)	**Mean (Ph.Eur.-u/ g)**	Stdev (Ph.Eur.-u/ g)	CV (%)
0	23	1.7	7.6	18	0.1	0.4	19	0.0	0.2
1	34	0.7	2.0	22	0.5	2.3	25	0.9	3.6
2	51	2.6	5.1	33	1.2	3.5	41	2.3	5.6
3	67	3.4	5.0	58	1.0	1.7	60	0.0	0.1
4	83	1.0	1.2	68	3.5	5.2	70	1.1	1.5
5	85	0.2	0.2	74	1.1	1.5	72	0.3	0.4
6	85	0.4	0.5	78	1.2	1.5	75	0.0	0.0
7	81	0.5	0.6	79	0.8	1.0	77	0.2	0.3
8	80	0.2	0.3	77	0.2	0.3	67	0.1	0.2
9	-	-	-	-	-	-	72	0.2	0.3

Active Pharmaceutical Ingridient (API)

Table A116. Relative **amylase** activities (Ph.-Eur.-u/ g) in several API samples were determined by ultrasonic method (URT/ HR-US) using one-point and three-point-calibration. Ultrasonic velocity was measured at 37°C after adding Amylase solution (URT/ HR-US) to starch solution 3 % (URT/ HR-US) in the sample cell or using a pre-mix. Shown are means of 2 sample solutions with 2 replicates each.

	One- point-calibration			Three-point-calibration		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)
Sample a	92,920	1,450	1.6	93,851	1,852	2.0
Sample b	105,159	1,347	1.3	107,968	1,485	1.4
Sample c	104,576	74	0.1	107,317	28	0.0
Sample d	100,594	660	0.7	102,707	667	0.7
Sample e	99,235	387	0.4	101,411	1,726	1.7
Sample f	98,736	2,382	2.4	100,547	3,394	3.4
Sample g	98,998	914	0.9	99,463	170	0.2
Sample h	98,072	1,415	1.4	99,895	2,294	2.3

Table A117. Relative **free protease** activities (Ph.-Eur.-u/ g) in several API samples were determined by ultrasonic method (URT/ HR-US) using one-point and three-point-calibration. Ultrasonic velocity was measured at 37°C after adding Free protease solution (URT/ HR-US) to Casein solution 1.25 % (URT/ HR-US) in the sample cell or using a pre-mix. Shown are means of 2 sample solutions with 2 replicates each.

	One- point-calibration			Three-point-calibration		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)
Sample a	4,518	75	1.7	4738	81	1.7
Sample b	4,698	13	0.3	4938	24	0.5
Sample c	4,778	175	3.7	4898	391	8.0
Sample d	4,652	18	0.4	4753	171	3.6
Sample e	4,589	119	2.6	4692	329	7.0
Sample f	4,551	12	0.3	4651	183	3.9
Sample g	4,954	510	10.3	5090	767	15.1
Sample h	4,650	40	0.9	4754	249	5.2

Table A118. Relative **total protease** activities (Ph.-Eur.-u/ g) in several API samples were determined by ultrasonic method (URT/HR-US) using one-point and three-point-calibration. Ultrasonic velocity was measured at 37°C after adding Total protease solution (URT/ HR-US) after activation with enterokinase to Casein solution 1.25 % (URT/ HR-US) in the sample cell or using a pre-mix. Shown are means of 2 sample solutions with 2 replicates each.

	One- point-calibration			Three-point-calibration		
	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)
Sample a	5,740	121	2.1	5,725	109	1.9
Sample b	6,328	147	2.3	6,208	128	2.1
Sample c	5,992	60	1.0	5,931	51	0.9
Sample d	5,950	120	2.0	5,906	96	1.6
Sample e	5,888	37	0.6	5,849	24	0.4
Sample f	6,008	96	1.6	5,950	74	1.3
Sample g	5,950	174	2.9	5,902	142	2.4
Sample h	5,856	33	0.5	5,830	26	0.5

Table A119. Relative **lipase** activities (Ph.-Eur.-u/ g) in several API samples were determined by ultrasonic method (URT/ HR-US) using one-point and three-point-calibration. Ultrasonic velocity was measured at 37°C after adding Lipase solution (URT/ HR-US) to Olive oil emulsion (URT/ HR-US) in the sample cell or using a pre-mix. Shown are means of 2 sample solutions with 2 replicates each.

	One- point-calibration			Three-point-calibration		
	Mean (Ph.Eur.-u/ g)	**Stdev (Ph.Eur.-u/ g)**	**CV (%)**	**Mean (Ph.Eur.-u/ g)**	**Stdev (Ph.Eur.-u/ g)**	**CV (%)**
Sample a	98,924	140	0.1	100,561	388	0.4
Sample b	99,974	583	0.6	102,911	548	0.5
Sample c	101,075	771	0.8	103,250	1579	1.5
Sample d	96,608	909	0.9	98,890	2116	2.1
Sample e	94,547	556	0.6	96,473	792	0.8
Sample f	94,436	1087	1.2	96,409	1483	1.5
Sample g	95,005	675	0.7	96,888	1559	1.6
Sample h	92,528	654	0.7	92,682	1193	1.3

Appendix A XXIV

Drug product III - VIII

Table A120. Relative **amylase** activities (Ph.-Eur.-u/ g) in several Drug product batches were determined by ultrasonic method (URT/ HR-US). Ultrasonic velocity was measured at 37°C after adding Amylase solution (URT/ HR-US) to starch solution 3 % (URT/ HR-US) in the sample cell or using a pre-mix. Shown are means of 2 sample solutions with 2 replicates each.

	HR-US			HR-US FTS		
Drug product	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)
III	41,490	253	0.6	-	-	-
IV	41,490	1,162	2.8	-	-	-
V	44,072	7	0.0	44,816	817	1.8
VI	43,349	1,470	3.4	44,010	169	0.4
VII	40,378	1,811	4.5	44,598	143	0.3
VIII	-	-	-	44,261	242	0.6

Table A121. Relative **free protease** activities (Ph.-Eur.-u/ g) in several Drug product batches were determined by ultrasonic method (URT/ HR-US). Ultrasonic velocity was measured at 37°C after adding Free protease solution (URT/ HR-US) to Casein solution 1.25 % (URT/ HR-US) in the sample cell or using a pre-mix. Shown are means of 2 sample solutions with 2 replicates each.

	HR-US			HR-US FTS		
Drug product	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)
III	2,434	3	0.1	-	-	-
IV	2,410	47	2.0	-	-	-
V	2,452	9	0.4	-	-	-
VI	2,526	35	1.4	-	-	-
VII	2,478	1	0.1	-	-	-
VIII	-	-	-	2,628	35	1.3

Table A122. Relative **total protease** activities (Ph.-Eur.-u/ g) in several Drug product batches were determined by ultrasonic method (URT/HR-US). Ultrasonic velocity was measured at 37°C after adding Total protease solution (URT/ HR-US) after activation with enterokinase to Casein solution 1.25 % (URT/ HR-US) in the sample cell or using a pre-mix. Shown are means of 2 sample solutions with 2 replicates each.

	URT (performed one year later)			HR-US FTS		
Drug product	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)
III	3,054	83	2.7	3,245	28	0.9
IV	2,962	65	2.2	3,144	62	2.0
V	2,798	72	2.6	3,057	16	0.5
VI	2,903	27	0.9	3,066	1	0.0
VII	2,881	123	4.3	3,099	32	1.0
VIII	2,989	30	1.0	3,134	2	0.1

Table A123. Relative **lipase** activities (Ph.-Eur.-u/ g) in several Drug product batches were determined by ultrasonic method (URT/ HR-US). Ultrasonic velocity was measured at 37°C after adding Lipase solution (URT/ HR-US) to Olive oil emulsion (URT/ HR-US) in the sample cell or using a pre-mix. Shown are means of 2 sample solutions with 2 replicates each.

	HR-US			HR-US FTS		
Drug product	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)	Mean (Ph.Eur.-u/ g)	Stdev (Ph.Eur.-u/ g)	CV (%)
III	60,781	955	1.6	-	-	-
IV	60,776	877	1.4	-	-	-
V	48,676	255	0.5	-	-	-
VI	47,938	19	0.0	-	-	-
VII	49,402	73	0.1	-	-	-
VIII	55,287	228	0.4	-	-	-

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